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Frontpage illustration: **Agar plate with bacterial cultures** (source: Dreamstime.com)

Preface

This thesis is submitted to the Technical University of Denmark in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry. The work was carried out from February 1st 2008 to February 1st 2011, at the Center for Microbial Biotechnology at the Department of Systems Biology under the main supervision of associate professor Thomas Ostenfeld Larsen (CMB) with associate professors Kristian Fog Nielsen (CMB) and Charlotte Held Gotfredsen (Department of Chemistry, DTU) as co-supervisors. The project was funded by the Programme Committee for Food, Health, and Welfare under the Danish Strategic Research Council.

First and foremost, I would like to thank my three supervisors for their scientific advice, constructive criticism, and support that extended beyond the confines of work. **Thomas**, your endless enthusiasm has always inspired and motivated me to stretch beyond my own expectations. **Kristian** and **Charlotte**, both with indispensable areas of expertise, have guided my work and provided pep talks at critical turns of the path. A special thanks to professor **Lone Gram** (National Food Institute, DTU) for mentoring me and always keeping her door open for invaluable discussions.

I am also grateful to; Laboratory technician **Hanne Jacobsen** for running a seemingly endless amount of MS samples for me and teaching me the ways of the TOF; Laboratory technician **Jette Melchiorsen** for cultivating massive batches of bacteria and screening hundreds of samples without batting an eye; My friend **Mette Lyngaae Rasmussen**, who worked at CMB for 6 months, keeping track of me and my samples. A special thank you to Dr. **Richard Kerry Phipps** for always knowing (…42!), and to all my other **wonderful colleagues** for providing me with food for thought, helpful ideas, and good laughs in equal measure! I would also like to thank a number of productive students, especially **Louise Kjærulff** who worked hard on isolating compounds from *Vibrio*.

In addition, I would like to extend my gratitude to the other **Galathea 3 partners** at the National Food Institute, KU-Life, and KU-Health for an interesting and educational collaboration. I greatly appreciate the opportunity to be involved in such a significant and fascinating project. To my fellow PhD students on the project, **Matthias** (thanks for sharing the dissertation journey with me!), **Nikolaj**, **Anita**, **Kristina**, and **Helle**: It has been a pleasure to share results, frustrations, and the excitement.

Preface

During my PhD I had the privilege to spend three months in the Marine Chemistry Group with professors **John Blunt** and **Murray Munro** at the University of Canterbury, New Zealand. Despite being on the brink of retirement, they took time to teach me all about CapNMR and share their wealth of experience. My stay in Christchurch was an experience for life in more than one way, and I still dream of the UoC campus coffee. Funding from Oticon, Otto Mønsted, and Knud Højgaards Foundation to finance my stay is greatly acknowledged.

I also had the great pleasure to participate in a number of international conferences, which have been a noteworthy source of inspiration and a great chance to present my own work. The marine natural products community is in many ways like a family, always ready to share 'tricks of the trade' with a young PhD student. I am very grateful to have had this opportunity to learn and develop friendships across oceans.

Credit is also due to the **Carlsberg laboratories** for allowing me to use their 800 MHz NMR spectrometer. It has been necessary!

Finally, I owe my gratitude to my family and friends for their boundless enthusiasm on my behalf and confidence in my ability to achieve my goals. To my husband **Dan** for his patience through my many travels, his encouragement, and his love that gives me the strength to walk on water. I could not have done this without you.

Kgs. Lyngby, February 1st, 2011

Maria Månsson

Summary

Bacteria produce a vast array of low-molecular-weight compounds endowed with a multitude of biological effects. As these compounds have developed in a biological context, it is hypothesized that bacteria exploit them to gain a competitive advantage for example through antagonistic interactions. It is speculated that these compounds can be used as antibiotics against biomedically important pathogens. The oceans, covering over 70% of the Earth's surface, constitute a unique ecosystem that promotes bacterial adaptations to the marine environment and provides a driving force for chemical diversity. Thus, marine bacteria represent a promising source of new chemical classes with antibiotic properties to counter the evolution of drug-resistant pathogens.

The aim of this PhD study was to explore a global collection of over 500 marine bacteria for their ability to produce bioactive natural products. This included both compounds with antibacterial activities and compounds interfering with virulent phenotypes of pathogens such as *Staphyloccocus aureus* and *Pseudomonas aeruginosa*. A primary goal was to provide a robust setup for chemical evaluation of the collected bacteria and develop methods that facilitate rapid dereplication of the associated extracts.

In order to efficiently evaluate the large collection of bacteria in terms of their biosynthetic potential and their ability to produce novel compounds, a combination of chemical profiling and dereplication techniques was applied. Profiling of the metabolites produced by members of *Pseudoalteromonas* and *Vibrionaceae* by LC-UV/MS was successful in differentiating strains at species and sub-species level. Within our collection, production of secondary metabolites was found to be independent of sample location, with 'strain siblings' from distant geographical locations producing the same combination of metabolites. However, significant intraspecies differences were still observed. In *Pseudoalteromonas luteoviolacea* and *Vibrio coralliilyticus* some of these differences were related to the production of antibacterial compounds. In this thesis, it is speculated that this contributes to specific niche-adaptation. The potential ecological role of antibiotics in *V. coralliilyticus* was further corroborated by studies showing that the antibiotic, andrimid, was produced under conditions mimicking the natural environment of this bacterium.

During the course of this PhD, three known antibacterial compounds were isolated: Indolmycin (*Pseudoalteromonas luteoviolacea*), andrimid (*Vibrio coralliilyticus*), and holomycin (*Photobacterium halotolerans*). All three are

examples of cosmopolitan antibiotics found in distantly related taxa. Genomic promiscuity among bacteria stresses the need for efficient and dynamic dereplication methods to avoid redundancy in isolation of bioactives. To extend our means of dereplication, an explorative solid-phase extraction (E-SPE) method based on three different ion-exchangers and a size-exclusion column was developed. E-SPE exploits the sensitivity of the biological assays to obtain information about the charge, polarity, and size of active components in a crude extract. This can be used to discriminate between possible candidates during dereplication and allows detailed mapping of bioactives.

This thesis provides a complete overview of known bioactive metabolites produced by members of the *Vibrionaceae* family, which constitutes an under-explored source of chemistry. Among the compounds reported so far, there is a prevalence of non-ribosomal peptides or hybrids hereof, with examples of N-containing compounds produced by NRPS-independent pathways. Compounds isolated from *Vibrio* and *Photobacterium* during this PhD corroborated this general trend. From *Photobacterium halotolerans*, we isolated two new cyclic tetradepsipeptides, designated solonamides A and B, as potent inhibitors of the *agr* QS system involved in virulence expression in *Staphyloccocus aureus*. Of special interest was a pronounced effect against a highly virulent, community-acquired, methicillin-resistant *S. aureus* strain (USA300).

In conclusion, this PhD study adds to the knowledge of marine bacteria as competent producers of secondary metabolites, some of which possess biological activities attractive for alternative strategies in antibacterial therapy.

Sammenfatning

Bakterier er i stand til at producere en bred vifte af små molekyler beriget med biologisk aktivitet. Da disse stoffer er udviklet i en naturlig kontekst, er forventningen, at bakterierne bruger stofferne som en del af konkurrencen mod andre bakterier. Derfor kan nogle af disse stoffer måske bruges som antibiotika mod sygdomsfremkaldende bakterier. Havet, der udgør over 70% af jordens overflade, er et unikt økosystem, der fordrer særlige tilpasninger hos bakterier, herunder kemisk diversitet. Havbakterier udgør således en lovende kilde til nye typer af kemiske strukturer med antibakterielle egenskaber, der kan bruges i kampen mod den stigende antibiotikaresistens.

Formålet med dette PhD studie var at udforske en global samling af over 500 havbakterier for deres evne til at producere naturstoffer med biologisk aktivitet. Dette gælder både stoffer med antibakteriel effekt og stoffer, der kan hæmme virulensen i vigtige sygdomsfremkaldende bakterier, såsom *Staphylococcus aureus* og *Pseudomonas aeruginosa*. Et af hovedmålene var at finde en konsistent fremgangsmåde til kemisk at evaluere de indsamlede bakterier og at udvikle metoder til at fremme dereplikeringen.

For effektivt at kunne bedømme det biosyntetiske potentiale i vores store samling af bakterier og deres evne til at lave nye stoffer, er der brugt en kombination af kemisk profilering og forskellige dereplikeringsteknikker. Kemisk profilering af stoffer produceret af bakterier tilhørende slægten *Pseudoalteromonas* og *Vibrionaceae* familien gjorde det muligt at skelne stammer på artsniveau samt at lave underinddelinger til disse. Produktionen af sekundære metabolitter i vores samling viste sig at være uafhængig af, hvor prøven var indsamlet, og nærtbeslægtede stammer indsamlet fra indbyrdes fjerntliggende dele af verden viste sig at producere de samme stoffer. Inden for samme art var der dog stadig signifikante forskelle. Blandt stammer tilhørende *Pseudoalteromonas luteoviolacea* og *Vibrio coralliilyticus* viste denne forskel sig at ligge i produktionen af antibakterielle stoffer. I denne afhandling foreslås det, at denne forskel er en del af bakteriernes tilpasning til forskellige økologiske nicher. Den potentielle økologiske betydning af antibakterielle stoffer for *V. coralliilyticus* blev underbygget af studier, der viste, at antibiotikummet andrimid blev produceret under betingelser, der mindede om de naturlige vækstbetingelser for bakterien.

Tre kendte antibakterielle stoffer blev isoleret under dette PhD studie, nemlig indolmycin (*Pseudoalteromonas luteoviolacea*), andrimid (*Vibrio coralliilyticus*) og holomycin (*Photobacterium halotolerans*). Alle tre stoffer er

eksempler på almindeligt udbredte antibiotika, der produceres af fjernt beslægtede bakterier. Det faktum, at nogle typer af antibiotika findes udbredt blandt bakterier, strammer kravet om mere effektive og dynamiske dereplikeringsmetoder, hvis man skal undgå at genopdage kendte typer af antibiotika. For at udvide vores arsenal af dereplikeringsmetoder, udviklede vi en 'explorative solid-phase extraction' (E-SPE) metode baseret på fastfase ekstraktionskolonner, herunder tre ionbyttere og en kolonne til størrelseskromatografi. E-SPE gav os muligheden for at udnytte den indbyggede følsomhed i vores biologiske test til at udvinde kemisk information omkring ladning, opløselighed og størrelse på de aktive stoffer i vores ekstrakter. Dette kan således bruges til at skelne mellem flere mulige kandidater under dereplikering og bruges til at give et klart overblik over aktive stoffer i samme ekstrakt.

I denne afhandling findes en komplet oversigt over kendte, biologisk aktive stoffer produceret af bakterier tilhørende *Vibrionaceae* familien, der i høj grad stadig repræsenterer en gruppe af uudforskede bakterier. Blandt de stoffer, der er fundet hidtil, stammer en overvejende del fra non-ribosomal petidsyntese samt andre typer af peptider. Stoffer isoleret i løbet af dette PhD studie tilhører ligeledes denne stofgruppe. Fra *Photobacterium halotolerans* isolerede vi to nye cykliske tetradepsipeptider, kaldet solonamide A og B. Disse stoffer hæmmede kraftigt quorum sensing systemet *agr*, der involveret i at regulere virulensen i *Staphylococcus aureus*. Solonamiderne viste sig at have en kraftigt hæmmende effekt på en særligt aggressiv, multiresistent *S. aureus* stamme (USA300).

Dette PhD studie bidrager til vores viden omkring havbakterier som kompetente producenter af naturstoffer, hvoraf nogle udviser biologiske aktiviteter, der er interessante i forbindelse med alternative behandlingsstategier af bakterieinfektioner.

List of original papers and other publications

Paper 1 **M. Månsson**, R.K. Phipps, L. Gram, M.H.G. Munro, T.O. Larsen, and K.F. Nielsen: *"Explorative Solid-Phase Extraction (E-SPE) for Accelerated Microbial Natural Product Discovery, Dereplication, and Purification"*, Journal of Natural Products (**2010**), 73:1126-1132

Paper 2 K.F. Nielsen, **M. Månsson**, C. Rank, J.C. Frisvad, and T.O. Larsen: *"Dereplication of Microbial Natural Products by LC-DAD-TOFMS: Experiences Gained from an Inhouse Database of 718 Mycotoxins and Microbial Metabolites"*, manuscript in preparation intended for Journal of Natural Products (**2011**)

Paper 3 N. Vynne, **M. Månsson**, K.F. Nielsen, and L. Gram: *"Bioactivity, Chemical Profiling, and 16S rRNA Based Phylogeny of Pseudoalteromonas Strains Collected on a Global Research Cruise"*, accepted for Marine Biotechnology (**2011**) DOI: 10.1007/s10126-011-9369-4

Paper 4 M. Wietz and **M. Månsson**, C.H. Gotfredsen, T.O. Larsen, and L. Gram: *"Antibacterial Compounds from Marine Vibrionaceae Isolated on a Global Expedition"*, Marine Drugs (**2010**), 8:2946-2960

Paper 5 M. Wietz, **M. Månsson**, and L. Gram: *"Chitin Stimulates Production of the Antibiotic Andrimid in a Vibrio coralliilyticus strain"*, to be resubmitted for Environmental Microbiology Reports after a favorable review (**2011**)

Paper 6 **M. Månsson**, A. Nielsen, L. Kjærulff, C.H. Gotfredsen, M. Wietz, H. Ingmer, L. Gram, and T.O. Larsen: *"Inhibition of Virulence Gene Expression in Staphylococcus aureus by Novel Depsipeptides from a Marine Photobacterium"*, ready-to-submit draft for Applied and Environmental Microbiology (**2011**)

Other papers (non peer review) in Danish and conference proceedings:

M. Johansen, N. Vynne, K.F. Nielsen, T.O. Larsen, and L. Gram: *"På fisketur efter nye bioaktive og bakteriehæmmende stoffer – en rapport fra verdenshavene"*, Dansk Kemi (**2008**), 89(11):12-17

M. Johansen, K. F. Nielsen, M.A.E. Hansen, E.K. Lyhne, T.O. Larsen, N. Vynne, J. Melchiorsen, and L. Gram: *"Dereplication Strategies for Discovery of Marine Microbial Natural Products"*, Poster at the Gordon Conference on Marine Natural Products (**2008**), US, February 24-29

M. Johansen, R.K. Phipps, L. Gram, T.O. Larsen, and K.F. Nielsen: *"E-SPE: Explorative Solid-Phase Extraction for Accelerated Natural Product Discovery and Purification"*, Poster at Nordic Natural Products Conference (**2009**), Iceland, June 2-5

M. Johansen, R.K. Phipps, L. Gram, T.O. Larsen, and K.F. Nielsen: *"E-SPE: Explorative Solid-Phase Extraction for Accelerated Natural Product Discovery and Purification"*, Poster at 6th European Conference on Marine Natural Products (**2009**), Portugal, July 18-24

M. Månsson, K.F. Nielsen, L. Gram, and T.O. Larsen: *"Exploring a Global Collection of Marine Bacteria for New Antibacterial Compounds"*, Oral presentation at MaNaPro XIII (**2010**), Thailand, October 16-24

Nomenclature

1D	One Dimensional
2D	Two Dimensional
ACD	Advanced Chemistry Development
Agr	Accessory Gene Regulator
AHL	Acylated Homoserine Lactone
AI	Autoinducer
AIP	Autoinducing Peptide
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photoionization
CA	Community Acquired
CapNMR	Capillary Nuclear Magnetic Resonance
Chr	Chromosome
CMB	Center for Microbial Biotechnology
DAD	Diode-Array Detection
DCM	Dichlormethane
DESI	Desorption Electrospray Ionization
DIMS	Direct Infusion Mass Spectrometry
DKP	Diketopiperazine
DMSO	Dimethyl sulfoxide
DQF-COSY	Double-Quantum Filtered Correlation Spectroscopy
DTU	Technical University of Denmark
EMEA	European Agency for the Evaluation of Medicinal Products
ESI	Electrospray Ionization
E-SPE	Explorative Solid-Phase Extraction

EtOAc	Ethyl Acetate
FAME	Fatty Acid Methyl Ester
FT-ICR	Fourier Transform Ion Cyclotron Resonance
GDSM	Genome Dedicated to Secondary Metabolism
GOLD	Genomes Online Database
HHa	3-hydroxyhexanoic acid
HILIC	Hydrophilic Interaction Chromatography
HOa	3-hydroxyoctanoic acid
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSL	Homoserine lactone
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Half maximal Inhibitory Concentration
Ile	Isoleucine
LC	Liquid Chromatography
Leu	Leucine
LH-20	Sephadex LH-20
MALDI	Matrix Assisted Laser Desorption Ionization
MAX	Mixed-mode Anion Exchanger
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MW	Molecular Weight
ND	Not Detected/Non Determined
NMR	Nuclear Magnetic Resonance

NRPS	Non-Ribosomal Peptide Synthase
NP	Natural Product
OSMAC	One Strain Many Compounds
PCA	Principal Component Analysis
Phe	Phenylalanine
PKS	Polyketide Synthase
PL	*Pseudoalteromonas luteoviolacea*
Pro	Proline
QS	Quorum Sensing
QSI	Quorum Sensing Inhibition
QSIS	Quorum Sensing Inhibitor Selector
RP	Reversed Phase
RNA	Ribonucleic Acid
RT	Retention time
SAX	Strong Anion Exchanger
SCX	Strong Cation Exchanger
SN	Supernatant
SPE	Solid-Phase Extraction
TDA	Tropodithietic acid
TLC	Thin-Layer Chromatography
TOF	Time-of-Flight
TTX	Tetrodotoxin
Tyr	Tyrosine
UHPLC	Ultra-High Pressure Liquid Chromatography
UoC	University of Canterbury
UV	Ultraviolet
Val	Valine

Table of contents

Background

The ability of nature to create unexpected chemistry has made natural products an unparalleled reservoir of chemical diversity, attractive for drug discovery and development. Although heavily studied over the past half-century, microorganisms continue to prove themselves to be reliable sources of novel bioactive compounds.

Today, many natural product-based antibiotics from the 'Golden Age of Antibiotics' in the 1950s and 1960s are rendered ineffective due to evolving resistance.[1] Motivated by this increasing need for new antibiotics, Center for Microbial Biotechnology and the National Food Institute participated in the global scientific expedition, Galathea 3, in order to mine the oceans for marine bacteria with antibacterial activity and potential biotechnological use. Throughout the nine month cruise, bacterial samples were collected from the seawater as well as various surfaces. This resulted in a unique collection of more than 900 different bacterial samples with the ability to inhibit the fish pathogen *Vibrio anguillarum*.[2] After re-culturing and re-testing, 519 strains were selected as representatives for all samples and geographical locations. Partial 16S rRNA gene sequence similarities revealed that the collection consisted of three major groups (Figure 1): *Vibrionaceae* (309 strains), *Pseudoalteromonas* spp. (128 strains), and the *Roseobacter* clade (29 strains).[2] All of these bacteria are Gram-negative bacteria, a group known to be less prolific in their production of secondary metabolites compared to e.g. actinomycetes.[3,4] For that reason, many species belonging to these taxa have never been investigated for their chemistry and therefore represent an under-explored source of new natural products.

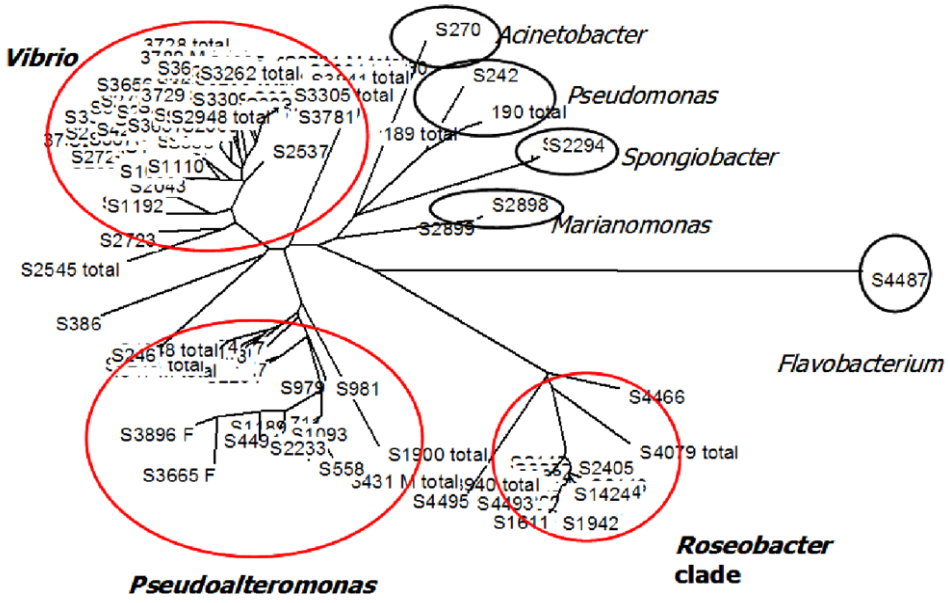

Figure 1: Cluster analysis based on 16S rRNA sequence homology of the 509 bacteria in the Galathea 3 collection.

To evaluate the collected bacteria, we set up a two-pronged screening approach, which was (*i*) to find new chemical classes with antibacterial properties, and (*ii*) to aim at new targets for antibacterial therapy.

In order to identify new chemical scaffolds, we sought to enhance the diversity of secondary metabolites by sourcing for new microbiological material. The marine environment, covering over 70% of the Earth's surface, is still largely unexplored and thus a major resource for the isolation of less exploited repertoires of microorganisms. By assessment of the global chemistry of a given bacterium, we aimed at selecting the strains representing the highest chemical diversity, at the same time avoiding redundant chemistry. In addition, we sought to develop a chemical screening strategy that allows efficient and accurate identification of interesting new compounds for purification. This would help us to identify compounds that have been overlooked previously.

In the last 10 years, there has been a shift from traditional growth inhibition towards new avenues in antibacterial control. With regard to the second component area, we aimed at discovery of compounds that do not necessarily kill pathogenic bacteria but rather inhibit virulence or biofilm formation, with the hypothesis that these compounds are not subject to resistance mechanisms.[5] Therefore, the screening array of the Galathea project included screening for

compounds that inhibit the quorum sensing response in pathogens like *Pseudomonas aeruginosa*[6] and *Staphylococcus aureus.*[7] Also, the screening setup included an immunoassay based on dendritic cells that identify compounds that can modulate an immune response.[8] This combination of assays provided a broad spectrum for screening our extracts.

The aim of this PhD study was to evaluate the chemical potential of the collected bacteria (**paper 3 and 4**), prioritize strains for chemical analysis by assessing their global chemistry and production of bioactive compounds (**paper 3, 4, and 5**), establishing suitable extraction, dereplication, and purification procedures (**paper 1 and 2**), and to purify active components for evaluation in the assays and structural elucidation (**paper 1, 4, and 6**). In this thesis, the results are presented and discussed in five different chapters. The first chapter introduces the potential and some challenges when dealing with marine microbial natural products. Chapter 2 outlines the experimental setup for the thesis. Chapter 3 describes and compares different dereplication strategies used through this study. Examples from both *Pseudoalteromonas* and *Vibrio* will be included in this chapter. Chapter 4 focuses on the chemistry of the *Vibrionaceae,* reviewing reported metabolites and discussing the biosynthetic potential of this under-explored group of bacteria. This chapter is intended as a mini-review for publication in a peer-reviewed journal; however, the version included in this thesis holds also unpublished results. Finally, chapter 5 is the overall discussion and conclusions.

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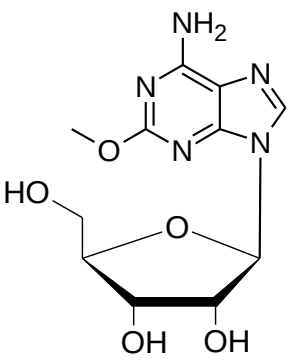
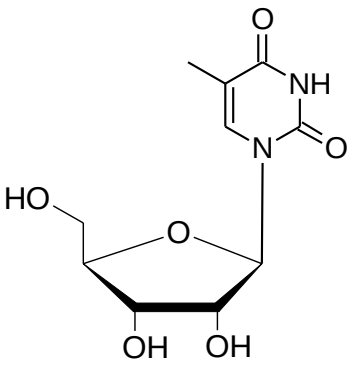
1 Marine Natural Products

Historically, the most important sources of natural products (or secondary metabolites) have been plants and terrestrial microorganisms. However, within the last 35 years,[1] the quest for novel chemistry has been extended into a world of fascinating marine biodiversity.[2,3] Some of the first marine organisms to be investigated for their production of secondary metabolites were plants and invertebrates. In 1951, the first marine-derived natural products were isolated from the sponge, *Tethya crypta*, that is the two nucleosides spongosine and spongothymidine.[4,5] Since then, natural products (NP) have been isolated from several marine invertebrates such as sponges, tunicates, molluscs, and bryozoans (Figure 1.1),[6] yielding a plethora of interesting bioactive molecules and covering new structural classes and functional groups.[7]



Spongosine



Spongothymidine

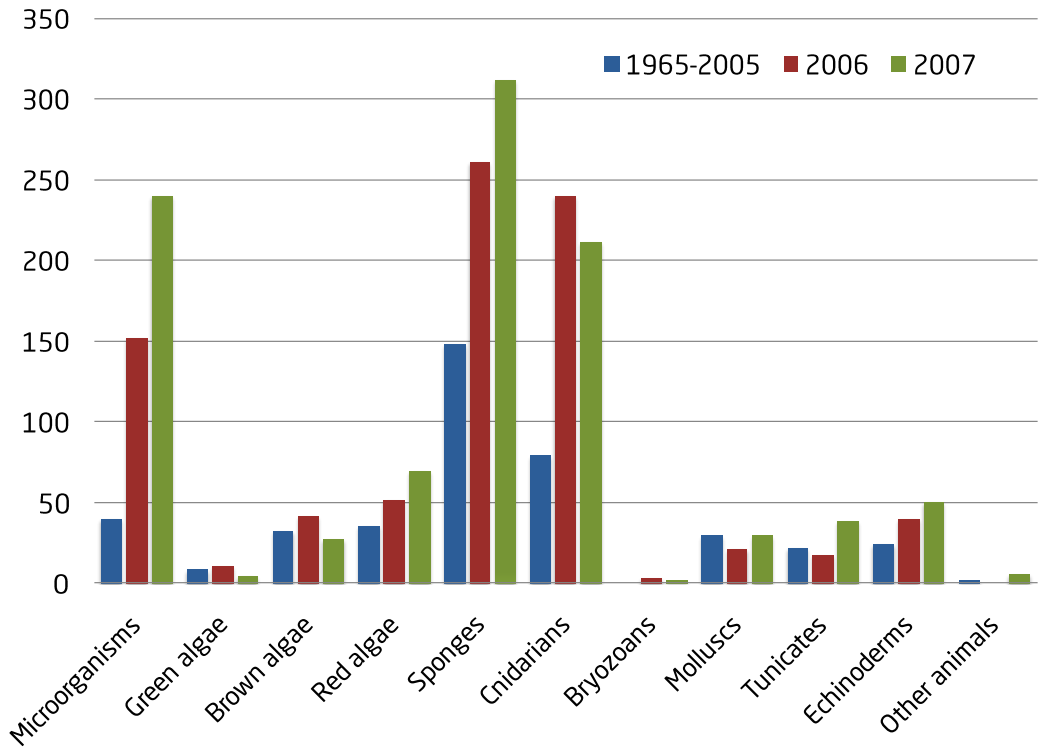


Figure 1.1 Distribution of marine natural products reported in MarinLit by taxonomic group. The data from 1965 to 2005 have been compiled and averaged. Figure adapted from Blunt et al. 2009.[8]

Many marine structures have served as leads for drug development.[9] Today, three marine-derived drugs are approved by the Food and Drug Administration (FDA).[10] That includes ziconotide (Prialt®, Elan Corporation) for chronic pain relief, the anticancer drug, cytarabine (Cytosar-U®, Bedford Laboratories/-Depocyt®, Enzon Pharmaceuticals), and the antiviral, vidarabine (Vira-A®, King Pharmaceuticals) (Figure 1.2). In addition, the anticancer drug, trabectedin (Yondelis®, PharmaMar) is approved by the European Agency for the Evaluation of Medicinal Products (EMEA) and is awaiting final approval by FDA.[11]

'The supply problem' has been a major obstacle for the use of drugs isolated from marine invertebrates,[6,12,13] as many of these organisms produce very low yields of their metabolites. In the case of trabectedin, the yield obtained from the source tunicate, *Ecteinascidia turbinata*, was less than 1 μg g^{-1}.[11] Owe to the structural complexity of many marine natural products, total synthesis of the compounds is not valuable. Striking structural similarities between metabolites from marine invertebrates and microorganisms raised questions about the true biosynthetic origin of these molecules.[14,15] Faulkner and coworkers (1993) were the first to show that sponge-derived metabolites were actually localized in the cells of a bacterial symbiont.[16] Demonstrating the correct origin of marine natural

products can be a quite comprehensive task that includes complete sequence analysis of the biosynthetic genes and heterologous expression to confirm their function.[17,18] Even so, today it is a well-acknowledged fact that many natural products of marine invertebrates are produced by associated microorganisms.[15,17] As production of complex molecules by microorganisms is a way to obtain a sustainable supply for marine drug development,[12] this has fueled the study of microbial natural products in the recent years. Within the last five years the number of metabolites from marine microorganisms has increased markedly,[8] and almost one fifth of new entries in AntiBase 2010 arise from marine bacteria.[19]

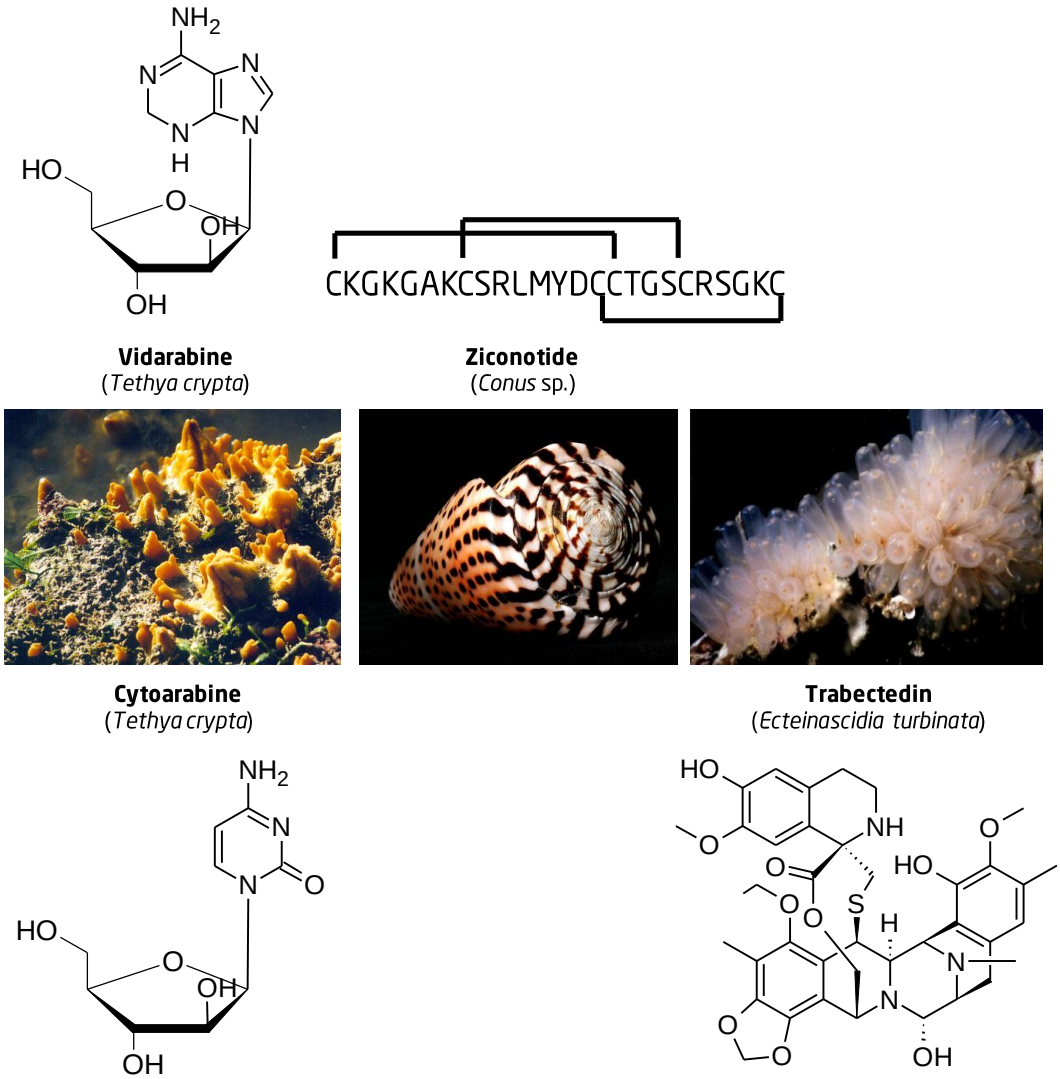


Figure 1.2 Marine natural products or derivatives thereof approved for use by the FDA or EMEA and their biological source. Vidarabine and cytoarabine are both synthetic nucleosides developed from the sponge metabolites, spongothymidine and spongouridine. Ziconotide is the synthetic equivalent of ω-conotoxin MVIIA originally isolated from the venom of the marine snail, *Conus magus*. Trabectidin is isolated from the sea squirt, *Ecteinascidia turbinata* (photo: PharmaMar).[10]

1.1 Bacterial sources of marine NPs

The marine environment, covering over 70% of the Earth's surface, is a vast and complex source of bacteria, especially considering that one milliliter of sea water contains more than 10^5 bacterial cells.[20,21] Notwithstanding, the undisputed and vital roles of bacteria in marine ecosystems like decomposition of organic matter and fixation of nitrogen, marine bacteria are still under-explored for their production of bioactive secondary metabolites.

Marine bacteria encompass many different phyla, including actinobacteria, proteobacteria, and cyanobacteria.[22] Divergence between terrestrial and marine bacteria is often defined as a sodium-specific requirement for growth;[7] however, the definition of a truly marine bacterium is difficult and many phyla contain both terrestrial and marine species.[23] Nonetheless, certain species and even genera, for example within the *Roseobacter* clade[24] and *Pseudoalteromonas*, have not yet been found in terrestrial environments.[25] Also, actinobacteria found in deep-ocean sediments seem to be autochthonous marine bacteria.[26,27] Even though bacteria do not keep to a strict definition of 'marine' and 'terrestrial' organisms, the oceans constitute a unique ecosystem that promotes bacterial adaptations to the marine environment.[23,28] Extreme variations in pressure, salinity, temperature, and nutrients create diverse micro-niches that can select for unique biosynthetic pathways leading to new types of molecules.[23] The best example of marine structural features is probably the bacterial production of halogenated, especially brominated, metabolites, due to the availability of these elements in seawater.[7,29]

The vast majority of marine bacterial species have unknown growth requirements and have not yet been cultured.[23,30] It is estimated that less than 1% of marine bacteria can be isolated using traditional culturing techniques,[20,21] in consequence leaving many natural products and their biosynthetic pathways inaccessible. So the full wealth of microbial diversity in the sea is yet to be revealed, and with it presumably an equally high chemical diversity.[31]

1.1.1 Isolated compounds from marine bacteria

The ability of marine bacteria to produce antibiotics was first documented in 1947 by Rosenfeld and ZoBell;[32] however, no compounds were isolated until 20 years later where Burkholder[33] and Lovell[34] purified and reported the structure of pentabromopseudilin (structure page 12) from *Pseudomonas bromoutilis* (later identified as *Pseudoalteromonas*). Since then, an increasing number of unusual secondary metabolites have been isolated from marine bacteria. Most reported metabolites originate from marine actinobacteria (Figure 1.3), mainly due to the proven success in their terrestrial counterparts.[35] However, members of *Alteromonas*/*Pseudoalteromonas* (genus revised in 1995 by Gauthier)[36] have also yielded a substantial number of metabolites with interesting biological activities.[37]

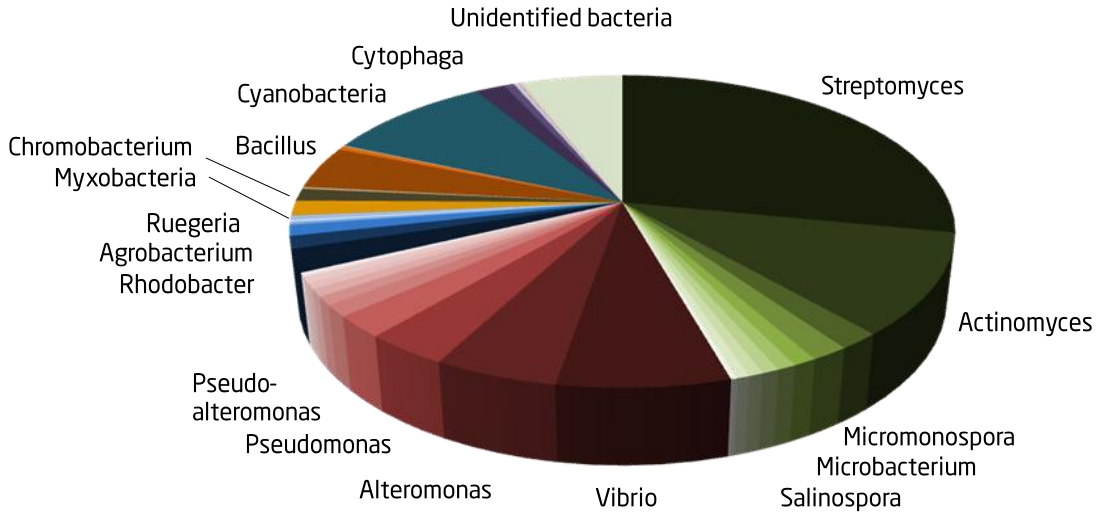
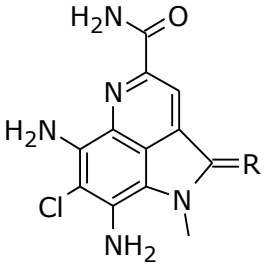


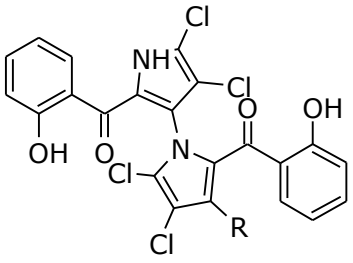
Figure 1.3 Distribution of reported marine bacterial metabolites in AntiBase 2010 according to their taxonomic origin.[19] Figure adapted and updated from Laatsch, 2006.[25] Green = actinobacteria, red = γ-proteobacteria, blue = α-proteobacteria, yellow = δ-proteobacteria, brown = β-proteobacteria, orange = Firmicutes, turquoise = cyanobacteria, purple = Bactiodetes. For the taxa including both marine and non-marine species, only compounds reported as from a 'marine' source are comprised.

Figure 1.3 displays the number of metabolites reported from marine bacteria in AntiBase 2010. Though the list of represented taxa might not be exhaustive, it gives a fair representation of the organisms of main interest to the natural product chemists so far. Aside from the groups highlighted in the figure, representing the highest number of compounds, there are examples of isolated compounds from other genera such as *Halomonas* and *Flavobacterium* (Appendix A). Hence, it is clear that many marine bacterial taxa have not yet been studied in detail for their production of secondary metabolites.

Actinobacteria have long been cultivated from marine samples;[38] however, these common soil bacteria were considered terrestrial contaminants, washed or blown into the sea. Improved sampling and cultivation techniques[39] revealed that actinobacteria can be found in abundance in ocean sediments [40] and on the surface of various marine macroorganisms.[41] Using culture-dependent analyses of 225 marine sediment samples, Jensen and Fenical (2007) found the actinobacterial diversity to span more than 22 different families.[42] Also, Schneemann et al. (2010) found more than 46 unique strains of actinobacteria, spanning five different genera, on the surface of a single sponge *Halicondria panacea.*[43] Although most species are not restricted to the sea, new species of indigenous marine actinobacteria have been described.[44] That includes *Salinibacterium amurskyense*[45] and *Serinicoccus marinus*[46] isolated from seawater, and *Salinispora arenicola* and *Salinispora tropica* from deep-sea sediment.[27] Actinobacteria boast some of the richest structural and biosynthetic diversity in the marine environment.[26,47] Compounds like the ammosamides[48] and marinopyrroles[49] produced by *Streptomyces* sp. derived from deep sea sediment samples have unique carbon skeletons and extraordinary biological activities. The ammosamides (A-B) are highly unsaturated aza-aromatic heterocycles with strong *in vitro* cytotoxicity against HCT-116 colon carcinoma (IC_{50} at 320 nM).[50] The marinopyrroles (A-F) feature an unusual *N,C*-linked bipyrrole decorated with several chlorine and bromine substituents.[51] The major analogues A and B displayed pronounced antibacterial activity against methicillin-resistant *S. aureus* (MRSA), with minimum inhibitory concentrations (MIC_{90}) of less than 2 μM.[49]



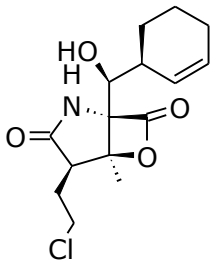
Ammosamide A, R = S
Ammosamide B, R = O



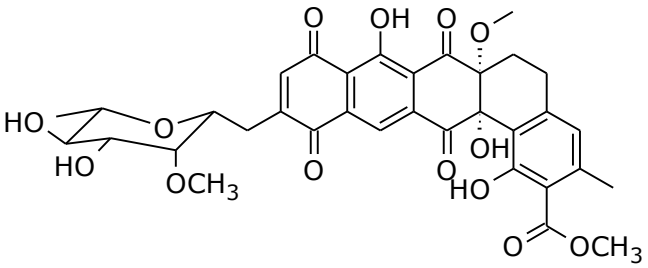
Marinopyrrole A, R = H
Marinopyrrole B, R = Br

The genus *Salinispora*, described by Jensen and Fenical in 2003,[52] was found to have great potential for the production of interesting bioactive metabolites.[53] Exploration of this new genus led to the isolation of the potent anticancer compound, salinosporamide A from *Salinispora tropica.*[54] The compound targets the 20S proteasome that induce apoptosis, and it has entered human clinical trials (NPI-0052, Nereus Pharmaceuticals) against solid tumors and lymphomas just

three years after its first discovery.[55] A strain of *S. arenicola* isolated from the tunicate *Ecteinascidia turbinata* was found to produce the benzo[α]naphthacene quinone antibiotic, arenimycin with marked activity against rifampin and methicillin-resistant *S. aureus* strains.[56]

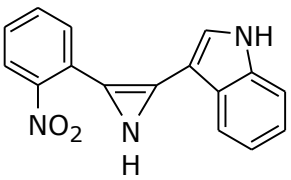


Salinosporamide A



Arenimycin

Most of the bacteria studied in this thesis belong to the γ-proteobacteria. Despite being the most represented bacteria in the culturable microbiota of many marine environments,[57-59] the number of compounds isolated is far smaller than that of marine actinobacteria. Most compounds isolated from γ-proteobacteria have been found in species belonging to the genera, *Alteromonas/Pseudoalteromonas* and *Vibrio.*[57] Like with actinobacteria, some unique structures with strong marine features have been isolated. Recently, a series of unusual nitro-substituted compounds called aqabamycins with antibacterial and cytotoxic effects were isolated from an unidentified *Vibrio* by the Laatsch group.[60]

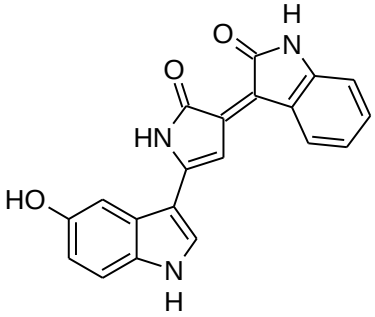


Aqabamycin H

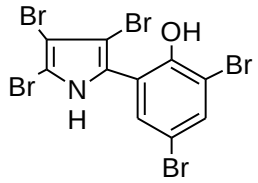
The chemistry of *Vibrio* will be more extensively reviewed in chapter 4.

Several members of *Pseudoalteromonas* are known to be producers of bioactive substances, many of which have been covered in reviews by Bowman (2007)[37] and Holmström et al. (1999).[61] As mentioned, the first antibiotic discovered from a marine bacterial source was pentabromopseudilin.[33,34] This highly brominated compound was found to possess strong antibacterial activity against a variety of human pathogens[62] including methicillin-resistant *S. aureus.*[63]

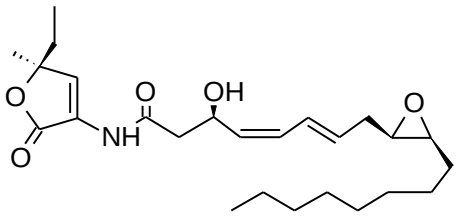
Since its first isolation in *P. bromoutilis*, it has been found in *P. luteoviolacea*[64,65] and terrestrial *Chromobacterium* sp.[62] Similarly, the purple pigment, violacein has been isolated from *P. luteoviolacea*,[66,67] *P. tunicata*,[68,69] *P. ulvae*,[70] and *C. violaceum*,[71] to name a few. Violacein presents diverse biological activities, including antimicrobial, antiprotozoan, antiviral, and anticancer.[72] Kjelleberg and co-workers (2008) posit that violacein plays a role in the grazing resistance of *Pseudoalteromonas* in the natural environment through induction of an apoptosis-like cell death mechanism in protozoan predators.[70] Other noteworthy compounds isolated from *Pseudoalteromonas* include korormicin.[73] This compound selectively inhibits the Na^+-translocating NADH:quinone involved in sodium transport in marine, halophilic Gram-negative bacteria, thus displaying a very narrow spectrum of activity.[74] *P. maricaloris* isolated from the sponge *Fascaplysinopsis reticulata* yielded a series of remarkable brominated penta-depsipeptides called bromoalterochromides.[75] Bromoalterochromide A was found to exhibit cytotoxic effects[76] but showed no antibiotic activity.[77]



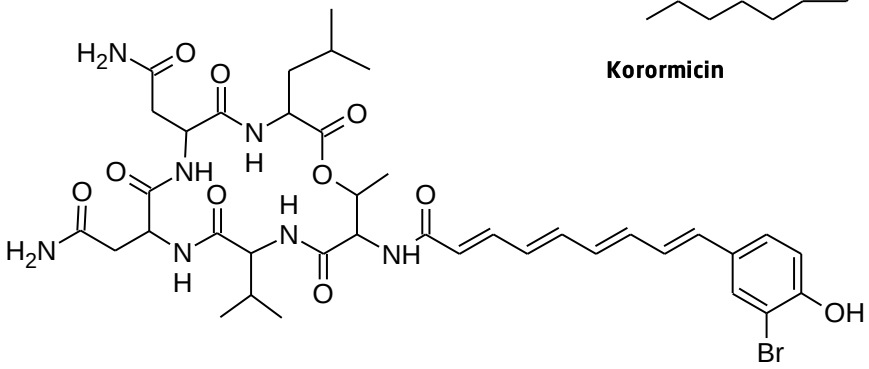
Violacein



Pentabromopseudilin

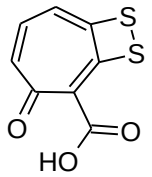


Korormicin

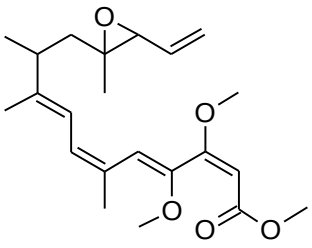


Bromoalterochromide A

Some of the more interesting sources of secondary metabolites among the α-proteobacteria are members of the *Roseobacter* clade, including *Ruegeria* and *Phaeobacter.*[78] These bacteria appear to be strictly marine and represent a significant fraction of marine bacterioplankton communities.[79,80] *Roseobacters* are often associated with different types of surfaces, especially marine algae, and the production of active secondary metabolites has been linked to the formation of biofilm.[81] Selected members of this group produce the antibacterial compound tropodithietic acid (TDA)[82] with an active four-membered sulfur-containing ring. TDA show broad but moderate antibacterial activity against both Gram-positive and Gram-negative bacteria. During the Galathea 3 expedition, strains belonging to the *Roseobacter* clade were collected from almost all geographical locations except those in Arctic and Antarctic waters. The majority of these strains were found to be *Ruegeria mobilis,*[21] which all were found to produce TDA.[83]



Tropodithietic acid

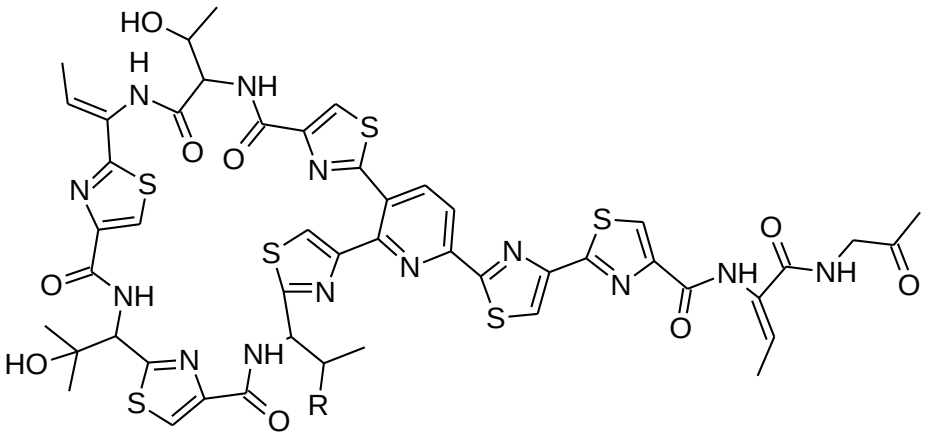


Haliangicin

Myxobacteria are gliding bacteria belonging to the δ-proteobacteria. Marine myxobacteria have been found in coastal sand or sediments,[84] biofilms in intertidal habitats,[85] and on surfaces of marine invertebrates and algae.[86,87] Marine members are differentiated from terrestrial myxobacteria mainly on the basis of halotolerance[88] and genera of true marine, halophilic myxobacteria include *Haliangium,*[89] *Plesiocystis,*[90] and *Enhygromyxa.*[87] There are only few reports of secondary metabolite from marine myxobacteria,[91] which is partly due to the only recent acknowledgement of these bacteria as true marine species and partly to their unculturable nature.[92] Haliangicin was the first compound reported from a marine myxobacterium, *Haloangium ochraceum.*[93,94] This compound displays potent antifungal activity but no effect against bacteria.

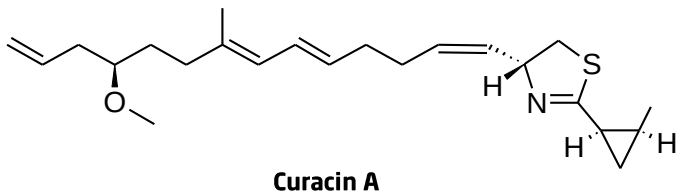
Within the *Firmicutes*, strains of the genus *Bacillus* represent the highest number of compounds reported from the marine environment. A large number of *Bacillus* species found in marine samples are also found in terrestrial environments; however, examples of halotolerant bacilli have been described including the genus *Halobacillus.*[95] Strains of *B. marinus, B. cereus, B.*

licheniformis, B. subtilis, and *B. pumilus* have been isolated frequently from marine invertebrates,[96-98] algae,[99] and sea water.[100] Marine bacilli have provided several peptide antibiotics. Two potent thiopeptide antibiotics, YM-266183 and YM-266184 were isolated from *B. cereus* from the sponge *Halochondria japonica.*[101,102] The compounds displayed activity against *Staphylococci* and *Enterococci* sp., including multiple-drug resistant strains.



YM-266183, R = OH
YM-266184, R = OMe

Cyanobacteria, or blue-green algae, are a group of filamentous bacteria. As these bacteria rely on photosynthesis, they are found only in the euphotic zone of the oceans.[103] Cyanobacteria represent a shallow clade of bacteria (taxonomy of cyanobacteria is currently under revision), even so with clear phenotypical differences. The cyanobacteria account for a substantial fraction of the reported metabolites from marine sources,[104,105] though it is difficult to completely separate freshwater and marine cyanobacteria.[104] A noteworthy example is curacin A,[106,107] an antitubulin agent that has been in preclinical trials[108] and served as lead structure for development of more potent synthetic analogues.[105,109]



Curacin A

1.1.2 Biosynthetic capacity of marine bacteria

Long have the central questions for natural product chemists been: what are the chances that we will find new and interesting compounds? What is the biosynthetic potential of a given organism? With the rise of the genomic era, the answers to these questions are now within reach. The cost of a full-genome sequence has drastically decreased and can be obtained quickly.[110] A high number of microbial genomes have been sequenced and even more are pending (Figure 1.4). That includes the genomes of many marine bacteria. Access to a full genome enables bioinformatic search for genes related to the biosynthesis of secondary metabolites, including clusters of polyketide synthases (PKSs), non-ribosomal synthases (NRPSs), and hybrids hereof, potentially linking them to production of specific compounds.

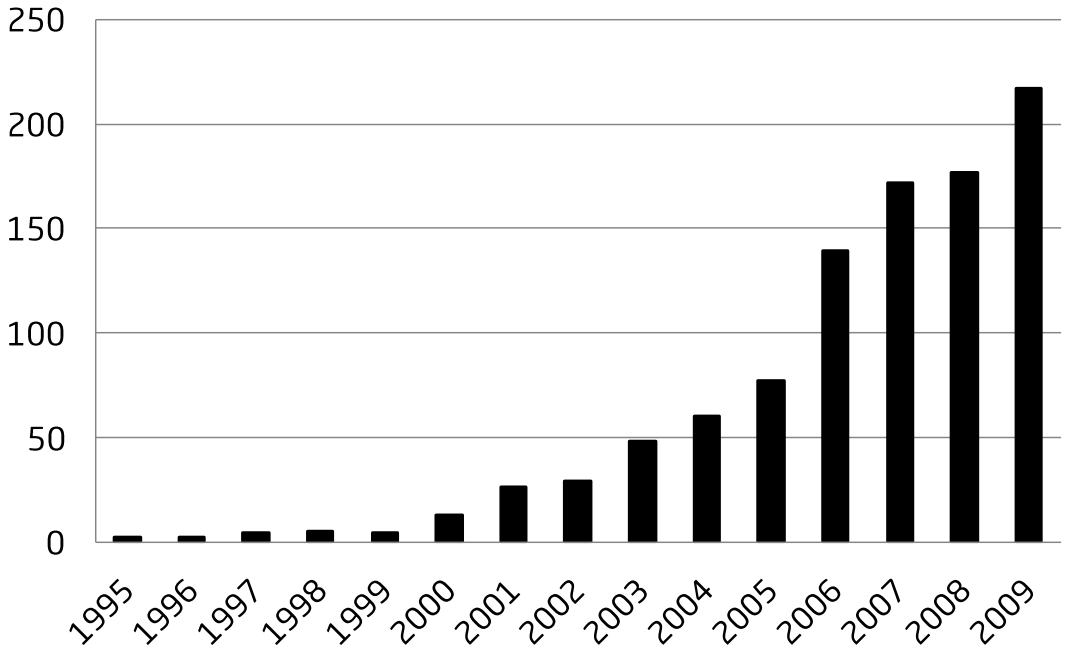


Figure 1.4 Number of completed bacterial genomes to 2009 (GOLD Stats).[111]

The metabolic capacity of the different bacterial genera varies greatly (Table 1). Statistical analysis of a subset of completed genomes revealed a strong positive correlation between genome size and secondary metabolite biosynthesis.[112] By studying functionally annotated genomes of some terrestrial and marine actinobacteria it becomes evident why these organisms have supplied such an extraordinary number of metabolites. The important producer of streptomycin and

other industrial antibiotics, *Streptomyces griseus* IFO13350 has no less than 34 gene clusters devoted to secondary metabolites in the 8.5 Mb genome, including 19 PKS/NPRS related clusters.[113] Despite having significantly smaller genomes, marine actinobacteria were found to match that capacity with 19 PKS/NRPS clusters in *Salinospora arenicola*, corresponding to an impressive 10.9% of the 5.8 Mb genome allocated to secondary metabolite production.[114,115]

Genomes of many marine bacteria have been investigated in order to understand their role in an ecological context, for example their role in CO_2 and N_2 fixation,[116,117] iron acquisition,[118,119] and environmental adaptations.[69,115,120,121] Whilst several members of *Pseudoalteromonas*[122,123] and *Vibrio*[121] have been sequenced, few have looked into the number of genes dedicated to production of secondary metabolites.[124] Thomas et al. (2008) investigated the genome of *Pseudoalteromonas tunicata* strain D2, and found only 2% of the genome allocated to production of secondary metabolites.[69] They were able to identify the genes responsible for the production of the known metabolites, violacein and tambjamine, and a single unidentified NRPS cluster. This scant evidence of PKS/NRPS related secondary metabolism was in accordance with the genomes of other sequenced *Alteromonas/Pseudoalteromonas* species, including *P. haloplanktis* TAC 125, *P. atlantica* T6c, and *Alteromonas macleodii* which all had less than 2% of their total genome allocated to secondary metabolism.[69] Whether this relatively low biosynthetic capacity is a common feature of γ-proteobacteria is yet to be revealed. However, the recent annotation of the genome of the marine shipworm symbiont, *Teredinibacter turnerae*, showed that this γ-proteobacterium might be able to "keep up with the Joneses" with at least nine biosynthetic clusters predicted to encode secondary metabolite pathways,[125] This bacterium has not been investigated for its ability to produce NPs and thus no compounds have been isolated so far. This underlines the pending potential for exploitation of marine bacteria for NP production.

The advances within genomics have revealed that bacteria rarely fulfill their potential for secondary metabolism under laboratory conditions.[126] In the wake of the availability of full bacterial genomes, mining for new chemical diversity has become widespread.[126-130] Before the sequencing of *Streptomyces avermitilis* in 2001, only three compounds were known, which was a number far smaller than the actual number of biosynthetic clusters.[47] Even today, only 13 of the total 37 secondary metabolite gene clusters in *S. avermitilis* are linked to production of a specific compound.[131] Thus, two thirds of the potential compounds produced by this organism is either not expressed or overlooked in chemical analyses. By exploring the genomes of other actinomycetes, for example *Salinispora*, it was found that their biosynthetic potential was underestimated with

up to 80-90% in some cases. Due to the modular, sequential design of some bacterial polyketides and non-ribosomal peptides,[132] it is possible to predict a putative structure *in silico* based on gene sequences with quite high accuracy. This can help unlock the nature of these cryptic pathways and identify their associated compounds.[126,133]

Species	Size	%GDSM	NRPS/PKS	Key metabolites	Reference
Streptomyces coelicolor A3(2)	8.7 Mb	~8%	11	Actinorhodin	Bentley 2002[134]
Streptomyces avermitilis	9.0 Mb	6.6%	16	Avermectin	Omura 2001[135] Ikeda 2003[136]
Streptomyces griseus IFO 13350	8.5 Mb	ND	19	Streptomycin	Ohnishi 2008[113]
Pseudomonas flouroscens Pf-5	7.1 Mb	~6%	9	Pyrrolnitrin Pyoluteorin	Loper 2007[137] Paulsen 2005[138]
Myxococcus xanthus DK1622	9.1 Mb	8.5%	18	Myxochromide Myxalamides	Goldman 2006[139]
* *Salinispora tropica* CNB-440	5.2 Mb	8.8%	15	Sporolide A, Salinosporamide	Udwary 2007[114] Penn 2009[115]
* *Salinispora arenicola* CNS-205	5.8 Mb	10.9%	19	Rifamycin Cyclomarin	Penn 2009[115]
* *Pseudoalteromonas tunicata* D2	4.9 Mb	~2%	3	Tambjamine Violacein	Thomas 2008 [69]
* *Teredinibacter turnerae* T7901	5.2 Mb	~7%	9	ND	Yang 2009[125]
* *Lyngbya majuscula*	8.5 Mb	~3%	ND	Curacin Barbamide	Jones & Monroe[140] MaNaPro XIII 2010

Table 1 Secondary metabolite biosynthetic capacity of selected full-genome sequenced bacteria. *Marine-derived bacteria. %GDSM = percentage of genome dedicated to secondary metabolism. NRPS/PKS = biosynthetic gene clusters related to NRPS, PKS, or NRPS-PKS hybrids. ND = no data.

Silent or 'cryptic' pathways may be expressed in the natural setting, e.g. in a more competitive environment;[141] however, turning these genes on in the laboratory can require the presence of different elicitors.[30,140,141] This be either stress factors, signal molecules, or culture conditions. Attempts to mimic the natural habitat can include cultivation in media based on natural seawater from the sample location, addition of sponge[142] or algal host extracts,[143,144] or marine-derived nutrients like chitin or alginate.[145] Okazaki et al. (1975) found that a marine actinomycete, *Chania* sp. only produced the antibiotic SS-228 Y[146] on media containing the seaweed, *Laminaria*.[144] A more random variation of culture conditions for metabolic 'talented' strains is the 'OSMAC' (One Strain MAny Compounds) approach that has the purpose of diversifying or maximizing the spectrum of

secondary metabolites from a given strain[147]. Attempts to trigger natural product biosynthesis also include biofilm formation.[148,149] Bruhn et al. (2007) found that production of the antibiotic TDA by members of the *Roseobacter* clade was closely linked to biofilm formation.[81] Production of antibacterial compounds has been linked to the production quorum sensing regulated homoserine lactones in several terrestrial bacteria,[150-154] and a similar connection have been suggested for production of TDA in *Roseobacter*[155] and violacein in *Pseudoalteromonas.*[156] Mixed fermentations or co-culture experiments can elicit secondary metabolite production in marine bacteria.[157] Mearns-Spragg et al. (1998) found that the production of antibiotics by surface-associated marine bacteria was conditional to the presence of competitors.[158] Angell et al. (2006) found two marine bacteria, a *Pseudomonas aeruginosa* and an *Enterobacter* sp., collected from the same sediment sample to have synergistic production of a blue pigment, pyocyanin, a compound not present in any of the pure cultures.[159]

Establishment of a meaningful link between secondary metabolite production and the true ecological setting of a bacterium will enable the identification of new targets for the NP chemists. Based on the number of isolated compounds, current marine hot spots for bioactive metabolite production are the actinobacteria[26] and cyanobacteria;[105,160] however, the chemical potential of many marine bacteria is yet to be unraveled and only by a combination of optimized NP discovery, genomics, and molecular biology will we find a way to isolate these compounds.[3]

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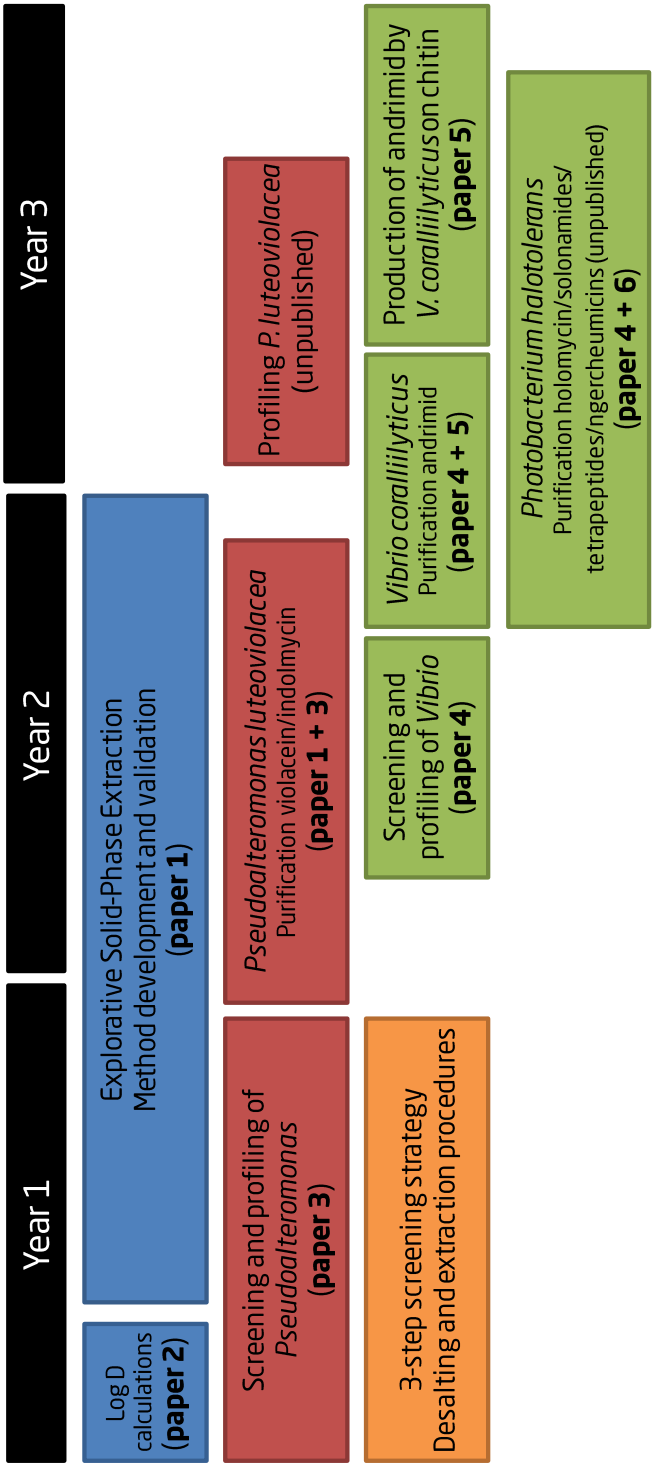
2 Overview experimental work

The figure is intended as an overview of the main experiments performed during the course of this thesis. The results obtained will be discussed as an integrated part of the following chapters. The experimental details can be found in the denoted papers.



3 Screening, profiling, and dereplication of natural products

Extracts from biological samples including microbial fermentates often represent a complex mixture of secondary metabolites covering a high structural diversity. A given extract usually attracts attention when an interesting biological activity, either from a drug discovery or an ecological perspective, is detected. To begin with, the chemical composition of a given extract is often completely unknown; including which compound(s) is responsible for the observed bioactivity. In order to identify the bioactive compounds, it is necessary to isolate and structure elucidate the individual components. In most cases, this poses a considerable challenge, requiring a great amount of time, labor, and cost. Thus, a primary goal for the natural product chemist is to reduce the time from the detection of biological activity to the identification a specific chemical structure.

The initial steps of this process will be described in the following sections.

3.1 The Galathea 3 setup

In order to efficiently evaluate the many bacterial strains collected during the Galathea 3 expedition while accelerating the discovery process, we have established a three-step chemical screening approach (Figure 3.1).

Our primary screen is based on raw extracts from less than 30 mL of bacterial culture, which is sufficient for screening in multiple bioassays and to obtain chemical profiles by LC-UV/MS, yet has the necessary scale to allow many samples to be processed per day. This allows us to quickly determine the spectrum of activity and biosynthetic potential of a given strain. The best candidates often display activity in more than one bioassay. In our collection, we have many strains that belong to the same species collected from distant geographical locations. Consequently, there is a high possibility of redundancy. To select the strains that will cover the broadest chemical diversity, thorough assessment of the global chemistry is necessary both in terms of quantity and novelty. Thus, careful dereplication by LC-UV/MS is performed at this level.

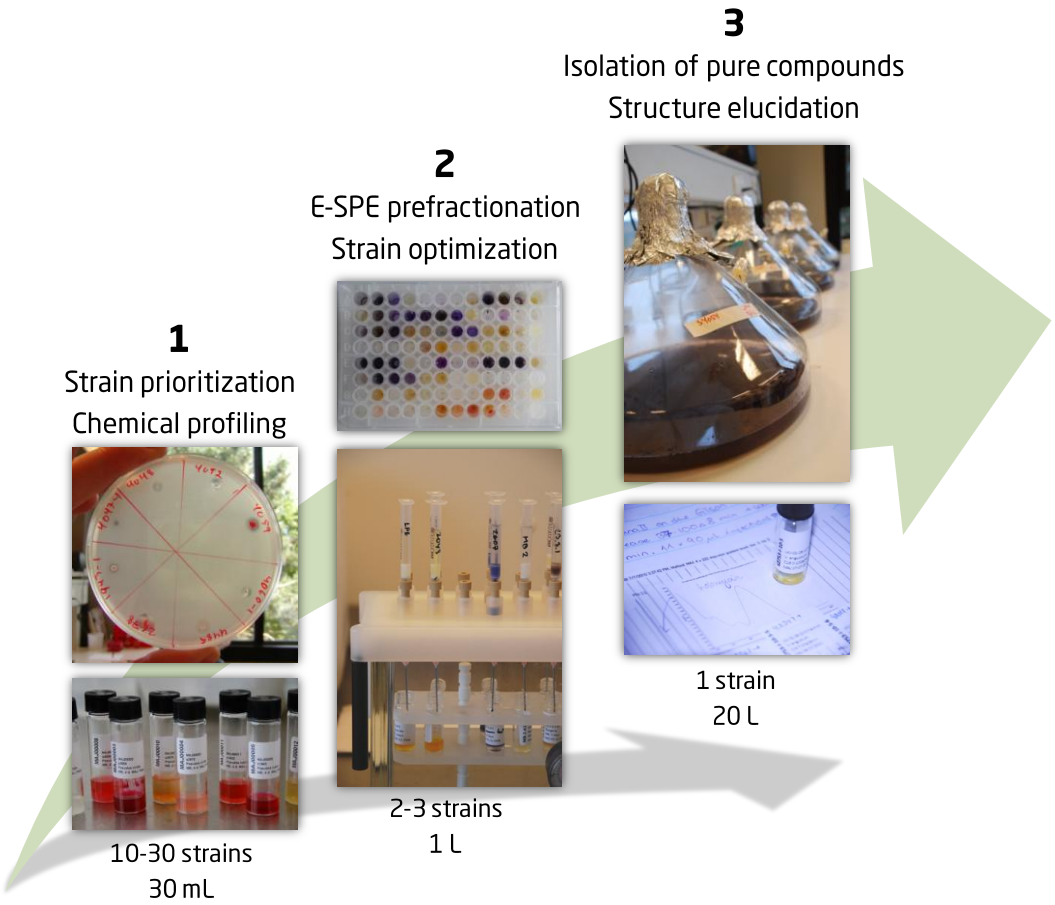


Figure 3.1 The Galathea 3 chemical screening approach, illustrating key steps from an active bacterial isolate towards the identification of the compound responsible for the observed bioactivity. (1) The first step is concerned with preliminary biological and chemical screening on a small scale; (2) an intermediate step is introduced to validate bioactivity of selected strains on a semi-large scale; (3) the best candidates for new chemistry and interesting biological activity are forwarded for large-scale purification and structural elucidation.

The most promising candidates from the primary screen are forwarded for a second level of screening and chemical evaluation prior to the investment in preparative-scale isolation. The bacteria are cultured in larger containers (200-1000 mL) in order to ensure that growth characteristics and metabolite production is reproducible. This indicates the yield that can be expected from a given organism when scaled-up. From these cultivations, the obtained biomass is sufficient for various pre-fractionations, including explorative solid-phase extraction (E-SPE) (**paper 1**). Fractions are evaluated in the various bioassays, which enables detailed mapping of the bioactive compounds. Quorum sensing inhibition assays are often less suitable for testing of crude extracts since the more subtle QSI effects are easily camouflaged by the presence of antibacterial compounds. Therefore, fractions are forwarded for QSI assays despite a negative response in the primary screen. The reduced complexity of the fractions compared

to the crude extract also facilitates a more detailed dereplication. On this scale, a purification strategy can be planned and tested in order to ensure maximum recovery and stability of active components.

Lastly, the best producers are selected for large-scale cultivation. We found that 10-20 L of culture was sufficient for most strains. For compounds excreted into the broth Diaion HP20 or XAD-7 was used for extraction and desalting. Compounds associated with the cells were extracted after harvesting the cells by centrifugation. As the typical yield of pure compounds obtained from the bacteria studied in this thesis was less than 0.5 mg L^{-1}, the majority of NMR data for structure elucidation has been obtained on either 800 MHz spectrometer with 3 mm cryo-probe (Carlsberg) or 600 MHz with a capillary flow-probe for enhanced sensitivity and improved resolution (for details on NMR refer to **papers 1, 4,** and **6**).

3.2 Chemical profiling of bacteria

Detailed chemical analysis of the small molecule composition (*the metabolome*) of an organism[1] can contribute to the understanding of phenotypic changes in an organism as response to its environment. Metabolite profiling (or chemical profiling) refers to the qualitative analysis of all metabolites produced with the objective to identify a specific metabolite profile that characterizes a given sample (Figure 3.2).[1] This can concern a group of metabolites related to a specific biosynthetic pathway or compound class (targeted metabolite profiling),[2] or completely unknown metabolites.[4] Even though primary metabolites are of great importance to the producing organisms, they are less useful for profiling purposes as they have a more general distribution and thus no differentiation power.[3] Therefore, within the scope of this thesis, metabolite profiling refers only to the analysis of the secondary metabolome.

Metabolite profiling involves peak/compound analysis, i.e. establishment of molecular formulas or other spectral features (Figure 3.2). Thus, metabolite profiling and dereplication are tightly coupled (section 3.3.1). A complementary approach is metabolite fingerprinting where 'patterns of metabolites' are investigated rather than the individual metabolites.[2] This can be used to cluster groups of extracts that represent different species or chemotypes in an unbiased way and to identify chemical markers responsible for the differentiation.[2]

In filamentous fungi, chemical profiling of secondary metabolites has been used with success, greatly improving fungal classifications and taxonomy.[3,5] Owe to a high degree of horizontal gene transfer, phylogenetically unrelated bacteria often produce the same secondary metabolites (**paper 1, 3, 4**). Concomitantly, bacteria within the same species tend to show a low degree of chemo-consistency, with very few metabolites, if any, consistently expressed in all members (**paper 3, 5**). Therefore, secondary metabolite profiling or fingerprinting is less suited for taxonomic purposes in bacteria. In a drug discovery or ecological context however, chemical profiling of bacteria very much has its justification as part of an intelligent screening strategy (**paper 4**).[5,6]

Definitions used in this chapter:

Metabolite profiling (*chemical profiling*): Qualitative and/or quantitative analysis of a large number of metabolites produced by an organism as seen by a specific methodology (sampling-extraction-analysis) (Villas-Bôas 2004)[1]

Metabolite fingerprinting: Unbiased, global screening approach to classify samples based on metabolite patterns (Dettmer 2007).[2] Metabolites not necessarily identified.

Chemo-consistency: Number of strains within a species that have the same metabolite profile (Frisvad 2008).[3]

Assessment of metabolite profiles or fingerprints can help select representatives of a species by recognizing redundant strains (strain dereplication) or strains of the same species that might produce different metabolites as a result of different niche-specificity (ecotype identification).[5,7] Such analyses are imperative to uncover the full biosynthetic potential of a bacterium (**paper 3**). Chemical profiling can also be used to prioritize compounds for further investigation.[8] Principal component analysis (PCA) of metabolite fingerprints can help identify peaks or compounds that are characteristic of specific chemotypes, and therefore likely to be of ecological importance. Also, profiles of secondary metabolites under different culture conditions[9] will help identify compounds that are involved in a particular phenotypic response (**paper 5**).

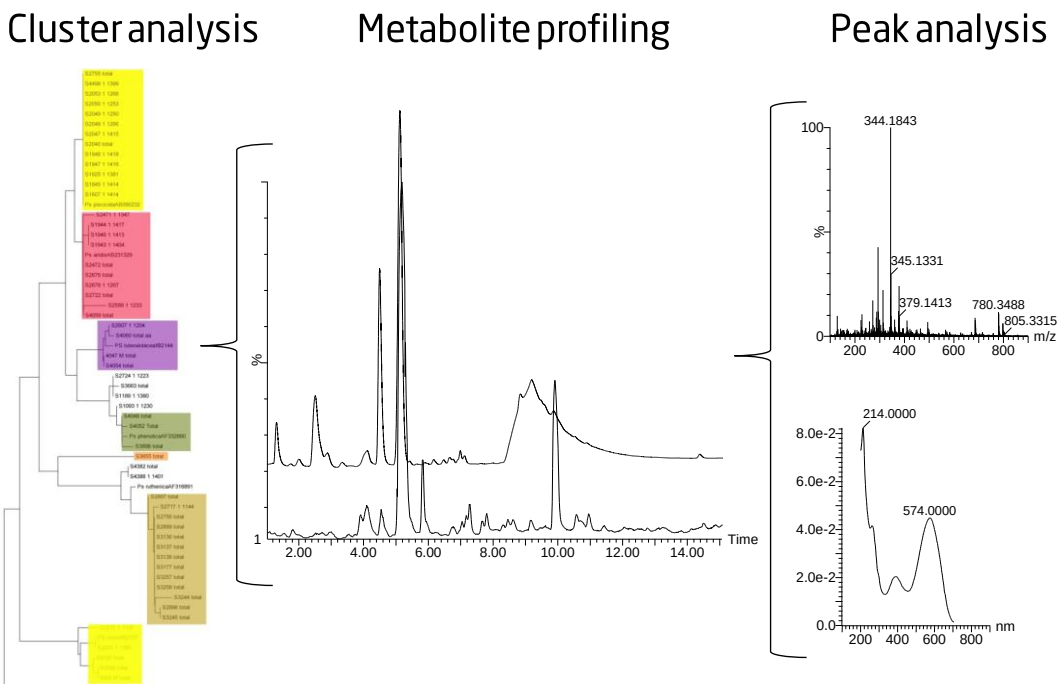


Figure 3.2 Illustration of the different levels in secondary metabolome analysis of a given organism. Metabolite fingerprinting uses patterns of metabolites to cluster groups of organisms. These groups are characterized by a specific metabolite profile, where the individual peaks can be analyzed in order to extract spectral data on individual compounds. The molecular mass and UV spectra can be used for subsequent dereplication.

3.2.1 Methods for chemical profiling

In many cases, the number of secondary metabolites produced by a single organism can exceed several hundred compounds with different physiochemical properties. This specifies the need for a versatile method that allows the detection of most compounds. At CMB, we use liquid-chromatography coupled with ultraviolet diode-array (UV/DAD) and electrospray ionization (ESI) mass spectrometry detection for the profiling of secondary metabolites. ESI has the ability to ionize a broad range of metabolites with minimal fragmentation which helps the interpretation of the spectral data.[9,10] Other interfaces suitable for metabolite analysis include atmospheric pressure chemical ionization (APCI)[11] and more recently, atmospheric pressure photoionization (APPI).[11-13] To obtain the best metabolite coverage, ionization is performed in both positive and negative mode (**paper 2**)., and complementary detection by UV and MS reduces the risk of overlooking metabolites that lack a chromophore or ionize poorly. Coupled to a LC system, the number of metabolites detected is maximized due to reduced signal suppression.[14] Reversed-phase (RP) chromatography is a versatile method for the separation of secondary metabolites, but also aqueous normal phase

systems such as hydrophilic interaction chromatography (HILIC) gain a foothold in metabolite profiling.[15,16] However, the latter is less suited for marine bacteria due to high levels of salt in the extracts which impairs binding to the column. The introduction of ultra-high pressure liquid chromatography (UHPLC) using sub-2-μm particles has greatly improved performance in terms of analysis time, allowing sample runs with chromatographic separation in less than 5 minutes.[17,18]

For metabolite fingerprinting of bacteria, the method of choice has been matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS.[19] Bacterial fingerprints have been compared for taxonomic and diagnostic purposes,[20] for example to identify pathogenic strains of *Bacillus* and *Yersinia*.[21,22] Dieckmann et al. (2005 and 2009) successfully applied whole-cell MALDI-TOF MS fingerprinting on strains of *Vibrio*[23] and other sponge-associated marine bacteria.[24] Most examples of bacterial fingerprinting are based on small protein analysis (*m/z* 2,000-15,000 Da) rather than secondary metabolites; however, Esquenazi et al. (2008) demonstrated how whole-cell MALDI-TOF MS can be used to detect jamaicamide (*m/z* 589), curacin (*m/z* 374), and other low-molecular weight compounds in cyanobacteria.[25] This suggests that the method can be extended to cover secondary metabolites,[26-28] even though the matrix will cause interference in the small mass area (*m/z* <300, dependent on the type of matrix). Covering a lower mass range, direct infusion mass spectrometry (DIMS) has been identified as a very useful method for detailed metabolite fingerprinting of filamentous fungi.[29] There are only few examples of the application of DIMS to bacterial extracts or suspensions.[30] Vaidyanathan et al. (2001) were able to discriminate various *Bacillus* strains at species and sub-species level using ESI-DIMS analysis of whole-cell suspensions.[31] Also, cell-free supernatants of *Halomonas* sp. were clustered for preliminary phenotypic characterization using ESI-DIMS.[32] However, DIMS analysis suffers from a number of disadvantages compared to LC coupled MS analysis, the major issue being signal suppression due to co-injection of extract components.[2,14] Also, in our experience DIMS analysis is less suitable for marine-derived extracts due to the high levels of salt that block the cone even for organic extracts.

For marine extracts, an attractive alternative to DIMS is desorption electrospray ionization (DESI) MS. Like whole-cell MALDI, DESI-MS is able to record mass spectra of intact cells without sample preparation[33] but will cover a mass range as low as *m/z* 50-500.[34] As DESI is a soft ionization technique like ESI,[33] it will cause minimum fragmentation and studies have demonstrated that DESI provides comparable results to that of ESI-DIMS,[35] however with a much higher salt tolerance.[36] DESI-MS analysis has successfully been applied to intact bacteria for species and sub-species differentiation.[34,37,38]

A supplement to MS-based fingerprinting is nuclear magnetic resonance (NMR). One-dimensional proton (1D-^{1}H) NMR has been used extensively for fingerprinting of plant extracts,[39] where chemical shift values and coupling patterns are used for clustering. Unlike mass spectrometric methods, NMR fingerprinting provides structural information on functional groups and compounds classes. Kim et al. (2006) used ^{1}H NMR to discriminate whole-cell extracts of cyanobacteria,[40] and recently the approach was applied by Boroujerdi et al. (2009) to characterize temperature-dependent metabolite fingerprints of *V. coralliilyticus*.[41] One of the major drawbacks of ^{1}H-NMR fingerprinting is high signal congestion. The enhanced sensitivity of many NMR probes[42] has enabled the use of two-dimensional (2D) experiments for fingerprinting,[43] increasing the dispersion of resonances and thus information content of the spectrum. Often ^{13}C isotope labeling is used to increase sensitivity in heterologous experiments like HSQC,[44] However, NMR-based methods still suffer from intrinsically poor sensitivity, which means that the metabolome analysis is restricted to the subset of secondary metabolites that exceeds a concentration threshold.[39]

3.2.2 Chemical profiling of marine bacteria

Metabolite profiling of marine bacteria has only been employed in a few cases so far. To the best of my knowledge, the first example of metabolite profiling of a large collection of marine bacteria is the investigation of *Salinispora* by Jensen and Fenical (2007).[45] They found a clear link between phylotype and secondary metabolite production irrespective of geographical location in members of the genus *Salinispora*. Widely distributed strains of *S. arenicola* had consistent production of three core metabolites, rifamycin, staurosporine, and saliniketal, joined by a number of accessory metabolites potentially acquired by horizontal gene transfer.[45] The study confirms that secondary metabolite profiles in bacteria represent more than a strain-specific phenotype despite intra-species differences. In line with these results, Gram et al. (2011) found production of the antibiotic TDA in members belonging to the *Roseobacter* clade to be a conserved feature independent of sample location.[46]

We have extended the use of LC-UV/MS metabolite profiling to members of *Vibrionaceae* (**paper 4-6**) and *Pseudoalteromonas* (**paper 3**). In our Galathea collection of 101 *Pseudoalteromonas* strains, we were able to resolve distinct chemotypes at species and subspecies level (**paper 3**). Several compounds were exclusively produced by strains belonging to the same cluster defining core metabolites, whereas other compounds were produced across more than one

cluster. The antibiotic pigments violacein and prodigiosin (Figure 3.4) were found to be restricted to members of *P. luteoviolaceae* and *P. rubra*, respectively. Interestingly, while violacein seemed to be a core metabolite defining the species, prodigiosin production was only detected in five of nine *P. rubra* strains. We assume that this may be due to loss of the ability to produce the pigment or strain variations in growth requirements for production of prodigiosin, as we did not observe prodigiosin in the type strain where it was originally reported.[47,48] Some species were found to be very homogenous in their metabolite production despite geographically distant sample locations, for example *P. flavipulchra* and *P. piscicida* (Figure 3.3) where all but one produced large amounts of bromoalterochromides[49] and other brominated metabolites.

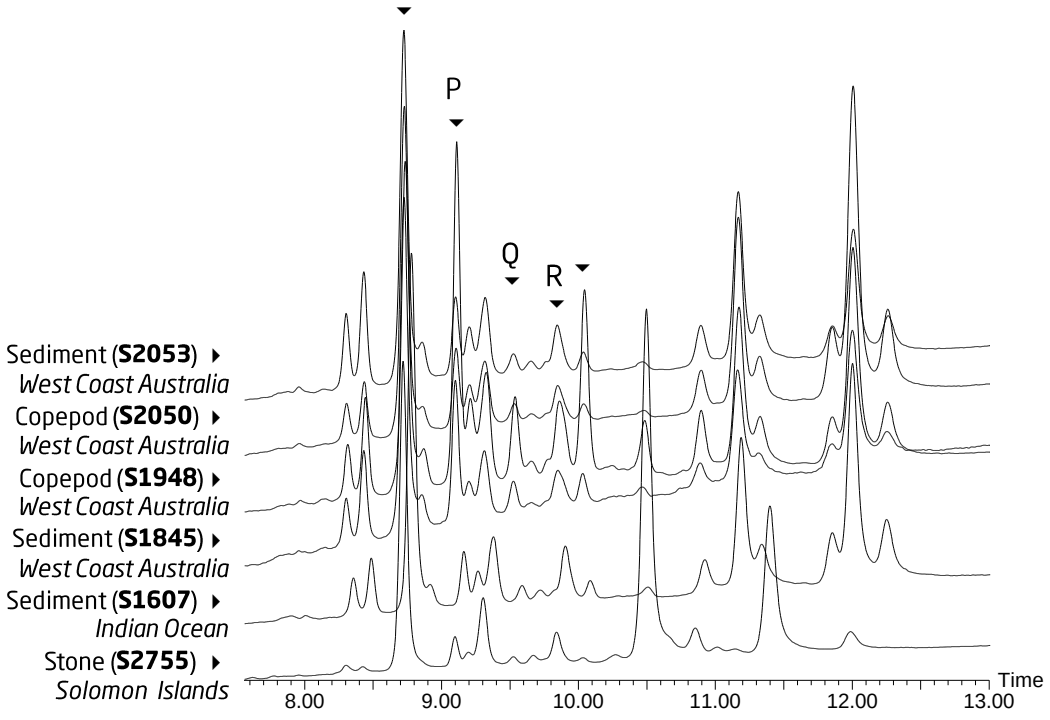


Figure 3.3 LC-UV traces of selected *P. piscicida/P. flavipulchra* strains with the source of origin. Brominated markers present in all investigated strains are marked with '▾'. P, Q, and R refer to tentatively identified bromoalterochromides (P = MW 843, Q = MW 921, R = MW 857) (**paper 3**).

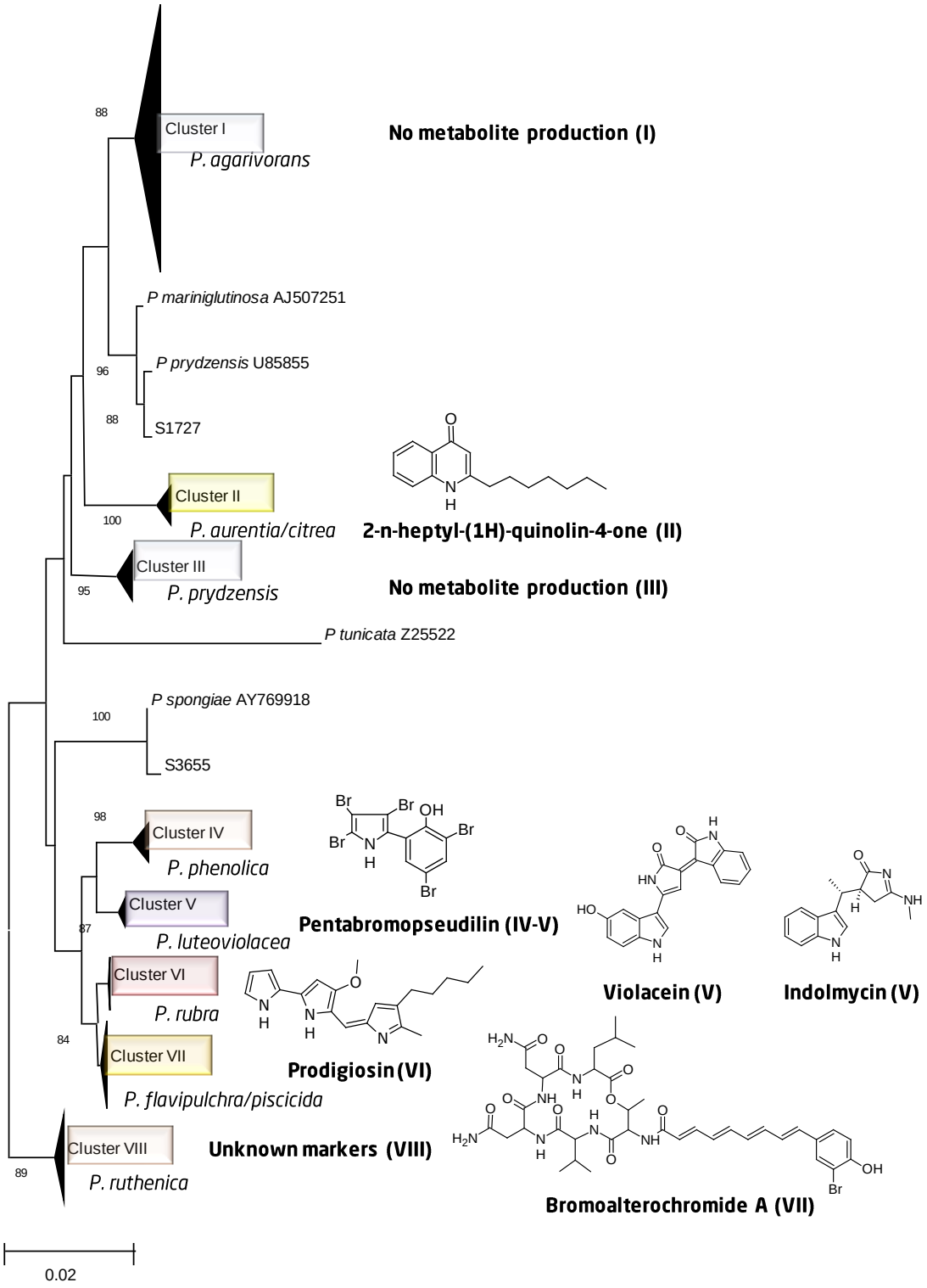


Figure 3.4 Phylogenic tree of the Galathea 3 *Pseudoalteromonas* strains based on 16S-rRNA sequence homology with denoted chemical markers representing known compounds. The scale bar relates to the number of amino acid substitutions per nucleotide position (as displayed by branch lengths in the phylogenetic tree). Clusters containing more than two non-type strain sequences were collapsed. Clusters are color-coded according to their overall pigmentation. For full figures and tables refer to **paper 3**.

In contrast, four *P. luteoviolacea* strains collected during the expedition showed both local and global variations in their secondary metabolite profile and antibiotic production. Chemotype PL1 (strains S2607/S4060) showed coordinated production of the antibiotics violacein and pentabromopseudillin (**paper 3**), while chemotype PL2 (S4047/S4054) produced violacein and indolmycin (**paper 1**) (see section 3.3.2). The grouping was supported by analysis of seven additional strains of *P. luteoviolacea* from culture collections (Månsson and Vynne, unpublished results, Table 1), adding at least one other chemotype, PL3, that produced violacein but neither indolmycin nor pentabromopseudilin (Figure 3.5). We hypothesize that the production of different antibiotic cocktails by *P. luteoviolacea* is a reflection of its adaptation to specific niches, for example as a successful colonizer of marine surfaces such as sponges or corals.[50,51]

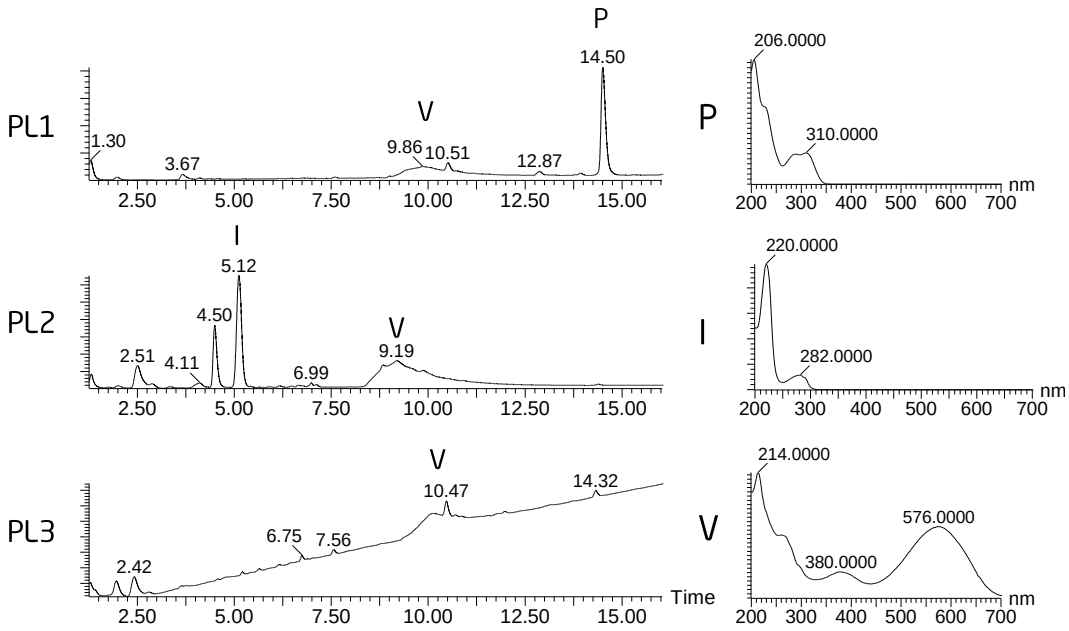


Figure 3.5 LC-UV metabolite profiles representing three distinct chemotypes within *P. luteoviolacea*. PL1 produce violacein (V, RT 9-10 min) and pentabromopseudillin (P, RT 14.50 min); PL2 produce violacein and indolmycin (I, RT 5.12); PL3 only produce violacein. Retention time of violacein changes due to precipitation during injection.

				Antibacterial compounds			Activity SN	
Chemotype	**Strain**	**Source**	**Origin**	V	I	P	*Vibrio*	*Staph*
PL1	DSM6061 (T)	Surface seawater	France, Mediterranean	+	-	+	+	+
	S2607	Lava rock	East Australia	+	-	+	+	+
	S4060	Seaweed	Costa Rica, Pacific Sea	+	-	+	+	+
	NCIMB 1944	-	France, Mediterranean	+	-	+	+	+
	CPMOR-2*	"Marine sample"	Possibly Mediterranean	+	-	+	+	+
	2ta16**	Coral	Florida Keys, Gulf of Mexico	+	-	+	+	+
PL2	S4047	Seaweed	Costa Rica, Pacific Sea	+	+	-	+	+
	S4054	Seaweed	Costa Rica, Pacific Sea	+	+	-	+	+
	CPMOR-1*	"Marine sample"	Possibly Mediterranean	+	+	-	+	+
PL3	NCIMB 1942	Sediment	France, Mediterranean	+	-	-	-	-
	NCIMB 2035	Surface seawater	France, Mediterranean	+	-	-	-	-

Table 1 Overview of *P. luteoviolacea* strains used for profiling, including their sample source and origin. Strains are grouped according to their chemotype as identified by their production of antibacterial compounds: Violacein (V), indolmycin (I), and pentabromopseudilin (P). Growth inhibitory activity of the supernatant (SN) against *V. anguillarum* (*Vibrio*) and *S. aureus* (*Staph*) is noted as well. Strains obtained culture collections except '*' from Prof. A. Sanchez-Amat (University of Murcia) and '**' from assistant prof. E. Allen (Scripps Institution of Oceanography).

A putative connection between chemotype and phenotype was also found among *Vibrionaceae*. Two strains of *V. coralliilyticus* (S2052/S4053) from distant sample locations displayed almost identical metabolite profiles, with the production of the antibiotic andrimid as a dominant feature (**paper 4**). Yet, andrimid was not observed in two closely related strains of *V. coralliilyticus*, including the type strain (**paper 5**). In addition, we found a temperature-dependent variation in the metabolic capabilities of S2052 and its relatives (**paper 5**). *V. coralliilyticus* S2052 had metabolic optimum at 25 ^{o}C, whereas the type

strain and its relative showed optimum at 30 °C. Interestingly, these two strains also show increased pathogenicity against corals at increased temperatures.[52] Thus, there might be a link between pathogenicity and metabolic capabilities.

When grown on the natural substrate chitin, *V. coralliilyticus* S2052 solely produced andrimid (**paper 5**) (Figure 3.6), indicating that andrimid serves a function while growing on chitin-containing surfaces such as zooplankton. In contrast, *P. halotolerans* S2753 sustained production of all metabolites including the antibiotic holomycin (**paper 4**). Furthermore, chitin stimulated the production of two potentially novel metabolites (RT 10.24 min, MW 618 and RT 11.15 min, MW 269) not observed on glucose. The different phenotypic responses to a natural growth substrate may reflect different niche-adaptations or different ecological functions of the compounds produced.

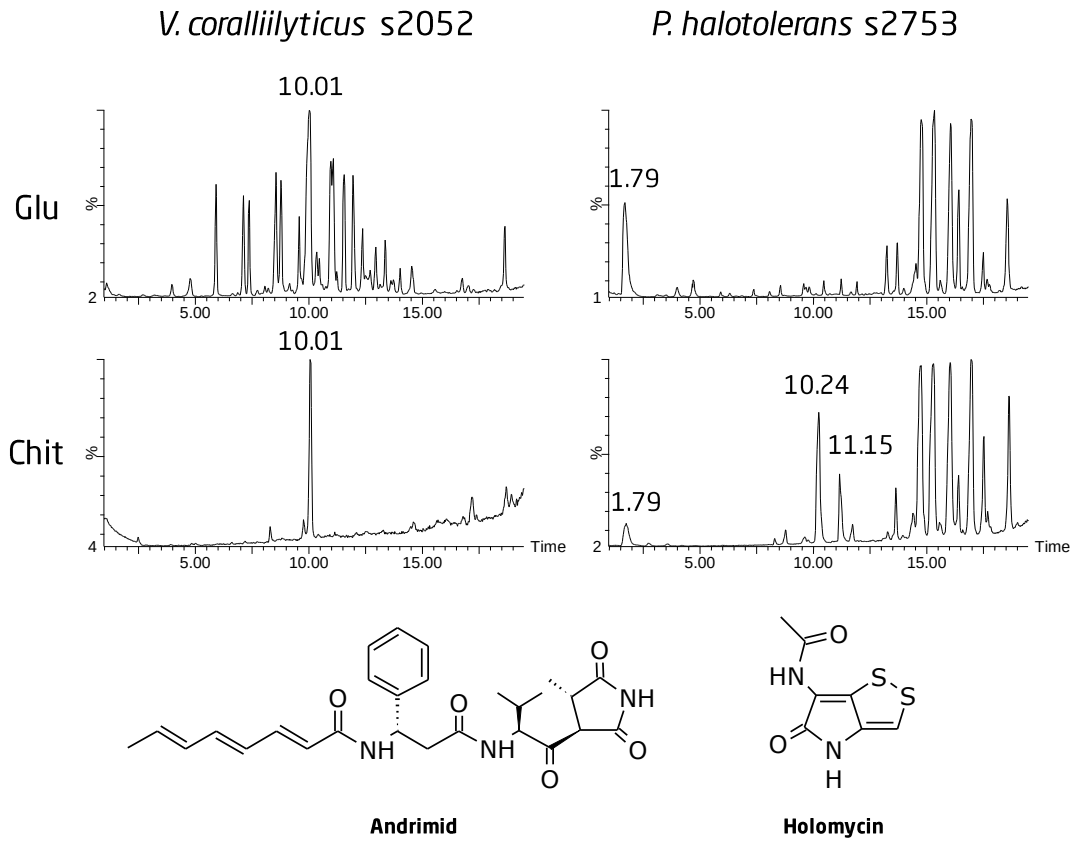


Figure 3.6 LC-MS metabolite profiles of *V. coralliilyticus* s2052 and *P. halotolerans* s2753 on marine minimal medium with glucose (Glu) and chitin (Chit), respectively. Production of secondary metabolites in s2052 is focused on andrimid (RT 10.01 min) when grown on chitin, while full secondary metabolite production including holomycin (RT 1.79 min) is sustained in s2753. Compounds observed only on chitin for S2753 (RT 10.24 and RT 11.15) does not match any known compounds in AntiBase 2010.

3.3 Dereplication

In practice, many isolated fractions of natural product extracts contain compounds that have been characterized previously. Due to the cosmopolitan occurrence of a lot of antibiotics and other bioactives, many compounds will be rediscovered even by intelligent selection of new types of organisms (**paper 4**).[53,54] Therefore, there is a continual need for dereplication in natural product discovery, i.e. the ability to exclude previously discovered compounds or classes of compounds.[55] Through rapid chemical and biological screening, dereplication provides fast, tentative identification of compounds in an extract, which makes it possible to rank natural product extracts according to the probability that the active constituent will be new.[56] By making dereplication an integral part of the early stages of the discovery process, it is possible to minimize the potential risk of replicating previous results, and thereby ensure that the time invested in isolation of compounds and ensuing structure elucidation is time well spent.[54,56]

Several approaches to the dereplication process exist, typically based on a combination of chromatographic and spectroscopic methods and database searching.[57] Traditional dereplication methods relied on thin layer chromatography (TLC) bioautography combined with numerous staining reagents for the identification of specific compound classes or functional groups.[55,58] Eventually, this approach was complemented by the use of HPLC-UV[59,60] for dereplication based on retention time and characteristic UV spectra. With on-line diode array detection (DAD) as standard equipment on most HPLC systems today, the UV spectrum of a compound still is one of the most readily accessible pieces of structural information.[5] Modern dereplication methods include various combinations of LC-UV, LC-MS, LC-MS/MS, and LC-NMR,[17] each with their set of advantages and limitations. Also, with the increasing availability of genomic data, dereplication performed on the basis of sequence information is emerging.[61] The dereplication methods applied during the course of this PhD at CMB and University of Canterbury will be discussed in the following sections.

For each analytical method, the data need to be translated into a searchable query for a database. A number of commercial databases are currently available for the marine natural products chemist:

- Encyclopedia of Marine Natural Products (8,700 compounds; J.-M. Kornprobst)
- Dictionary of Natural Products (230,000 compounds; J. Buckingham)
- Dictionary of Marine Natural Products (30,000 compounds; J. Blunt and M. Munro)

- AntiBase (37,000 compounds; H. Laatsch)
- MarinLit (22,000 compounds; J. Blunt and M. Munro)
- AntiMarin (50,000 compounds; J. Blunt, H. Laatsch, and M. Munro)

AntiMarin, being compiled from AntiBase and MarinLit, is currently the largest and most specialized resource for the dereplication of marine microbial natural products. The database contains the structures of all known compounds from marine macroorganisms and marine and terrestrial microorganisms. Besides accurate masses and UV characteristics, all compounds are coded for ^{1}H NMR-recognizable features, which make it possible to extend the query to include characteristic functional groups. The work at CMB is only concerned with investigation of marine microorganisms; therefore our database facilities are restricted to AntiBase.

Dereplication can be an laborious task, as each probable candidate from a database hit list need to be evaluated. In 1994, Corley and Durley estimated that it takes $50,000 and three months to isolate and characterize an active compound from a natural source.[62] For every known compound isolated, these resources are squandered. Even though this number might not be completely valid today due to improved equipment and techniques, it greatly exceeds the cost of subscribing to any commercial database. Thus, the time and effort spent on early dereplication is worthwhile.

3.3.1 Methods for dereplication

The method of choice for front line dereplication of extracts at CMB is LC-UV/MS (**paper 2**).[5,63] LC-MS is a simple and rapid dereplication method that can be controlled automatically to obtain a high throughput and reproducibility. Dereplication by this approach is based on determination of the accurate mass of the $[M+H]^+$, $[M-H]^-$, or other identified ions. Today, it is the most widely used dereplication method as accurate mass can be used as query in nearly all natural product databases.[54] LC-MS is a generally sensitive method, providing reliable results within the 10^{-15}-10^{-9} g range for most compounds, although some classes of especially very small molecules and non-oxygen and non-nitrogen containing compound often do not ionize (**paper 2**). The signals obtained can be easily deconvoluted, which makes LC-MS very suitable for the dereplication of complex mixtures.

Correct interpretation of LC-MS data is essential for correct assignment of the molecular mass and the subsequent database searching. ESI ionization is the most common ionization technique for LC-MS, and in most cases interpretation is relative simple (Figure 3.7). Other ions than the molecular ion can be quite abundant depending on the nature of the target compound, the ion source parameters (e.g. the applied skimmer voltage), and the solvents and additives used (**paper 2**). These ions can validate the assignment of the molecular ion, especially for compounds undergoing both fragmentation and adduct formation.[63] However, this part of the process is prone to error. Strong adduct ions such as $[M+Na]^+$, $[M+NH_4]^+$, $[M-H+HCOOH]^-$ etc. or fragment ions can easily be misinterpreted as $[M+H]^+$ or $[M-H]^-$, leading to false compound identification.[64,65] Interpretation of the data can be hampered by the presence of interfering ions from minor components more prone to ionization than the target compound. On the basis of statistical analysis of our in-house metabolite database, we found that ESI^+ alone was able to unambiguously assign the correct molecular mass for 56% of the compounds (**paper 2**). Extended with ESI^-, this fraction was increased to 93%, demonstrating the power of LC-ESI-MS.

The accuracy of the data is fundamental for the success of MS-based dereplication.[66] The American Chemical Society has defined 'accurate mass' as a mass measurement error of maximum 5 ppm. On most state-of-the-art mass analyzers operated under 'high-resolution' (HRMS) conditions, the mass accuracy is typically 1-5 ppm (depending on internal mass calibration), with some analyzers (FT-ICR) providing ultra-high resolution with sub ppm mass accuracy. However, few natural products laboratories currently have access to equipment of that accuracy. Therefore, it is common that several molecular formulas are compatible with the observed molecular mass.[66] This necessitates a relatively high tolerance in database queries, resulting in a lot of candidates of which most are false positives. Statistical analysis of all molecular formulas in AntiBase 2008 (**paper 2**) revealed at 5 ppm mass difference, 195 compounds with different elementary composition could not be differentiated from each other in the mass range 400-99 Da. The number of candidates can be reduced up to three fold when isotope ratios are used (Cl, Br and ± 2 carbons).

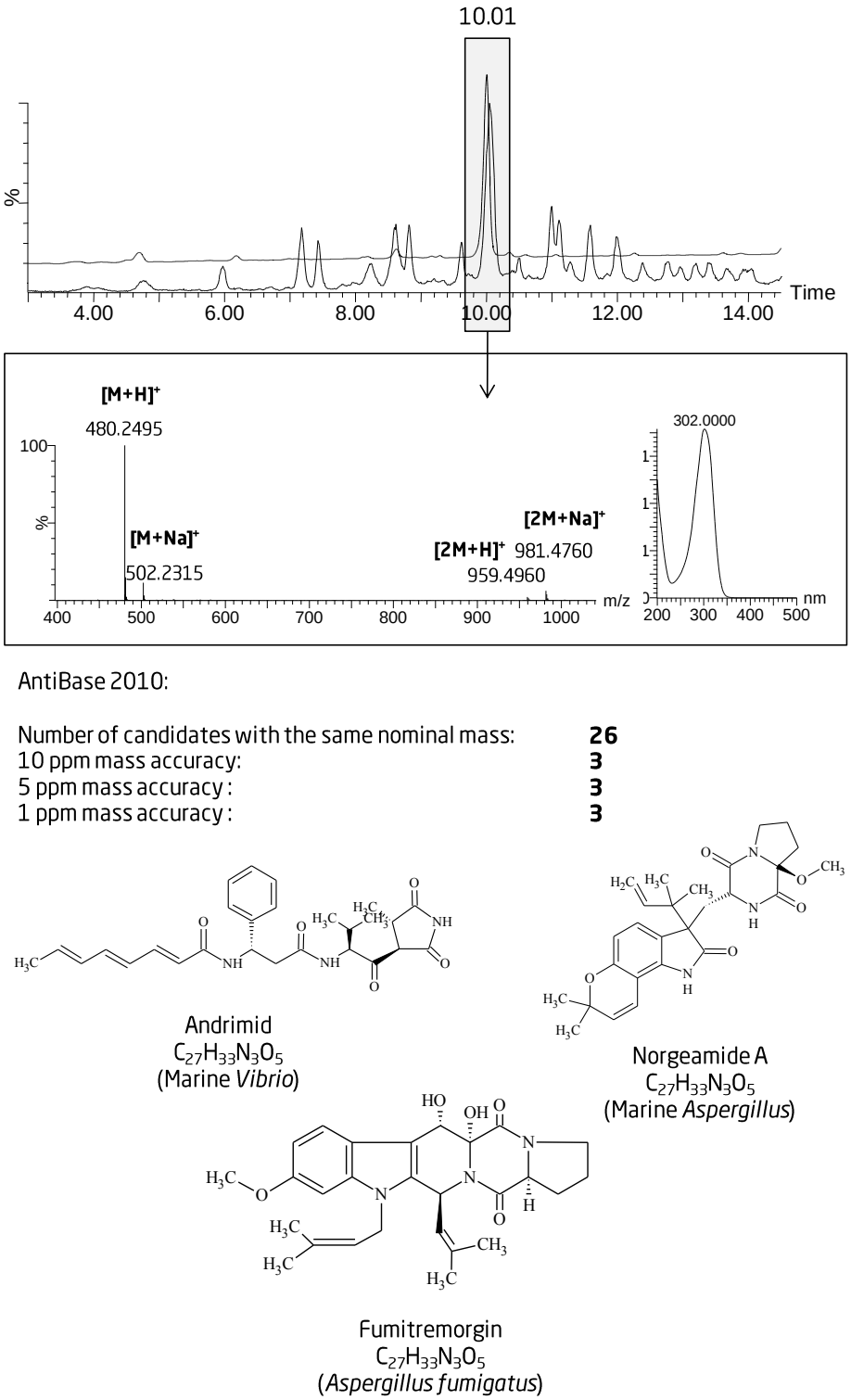


Figure 3.7 Dereplication of an extract of *Vibrio coralliilyticus* strain S2052 by LC-UV/HRMS. MS and UV spectra are extracted from the peak at RT = 10.01 min in the chromatograms. In the MS spectrum, the molecular ion is assigned based on the presence of an $[M+Na]^+$ adduct and the dimeric $[2M+H]^+$ and $[2M+Na]^+$. When used as query in AntiBase 2010, 26 candidates have the same nominal mass. When accurate mass is used, the number of candidates is reduced to three within 10, 5, and 1 ppm. All possible candidates have the same molecular formula of $C_{27}H_{33}N_3O_5$ and thus cannot be distinguished by MS alone. Andrimid is tentatively identified based on the taxonomic origin.

Below 1 ppm mass accuracy, molecular formulas could be unambiguously assigned for all compounds in AntiBase. Yet, this does not solve the problem of redundant molecular formulas. This problem can in part be circumvented by extended information gained by MS/MS.[67] On newer MS instruments, MS/MS spectra can be recorded automatically in data-dependent acquisition mode and the accurate mass of characteristic fragments provides direct structural information.[9] This is particularly useful for peptides and other biopolymers where sequences can be derived *de novo*. For small molecules, the fragment ion mass spectra are less consistent between different spectrometers and instrumental parameters. Several search algorithms[68] and commercial software packages such as the ACD/MS Fragmenter and Mass Frontier are available for *in silico* prediction of fragmentation patterns;[69] however, studies by Horai et al. (2009) on a subset of 453 metabolites in MassBank[70] show the confidence in peak annotation to be as low as 3%.[69] Thus, in order to make MS/MS data available for meaningful database queries, compound-specific reference spectra need to be collected at several different collision energies and spectra must be filtered for background noise before database storage.[71] Consequently, MS/MS for dereplication purposes is currently restricted to those compounds included in in-house libraries.[67]

MS in itself is not a very discriminatory method,[72] and therefore secondary information need to be used for exclusion of candidates during a database search. Comparison of UV data can be very useful as it will give indications towards the composition of a potential chromophore.[5,59] However, for non-chromophoric compounds this is of no value. Also, UV data is rarely complete in commercial databases and thus not available for comparison. Retention time (RT) is an additional parameter for dereplication.[73] For certain compound classes there are a good correlation between RT and logD values (**paper 2**). LogD values can be predicted, yet retention time is less suitable for a searchable query as even slight changes in the chromatographic conditions might change the RT considerably.[58] Information on the taxonomic origin of a given compound is often provided in commercial databases and can thus be used to assess the probability of a compound being known or novel (Figure 3.7). This information is of little value for the dereplication of bacterial extracts due to the high degree of horizontal gene transfer. Overall, unless data is compared to that of an authentic standard, MS-based dereplication will only provide a tentative identification of a compound (Table 2).

Within recent years, the combination of HPLC and NMR has emerged as an attractive analytical tool for dereplication of natural products aided by improved probe design and magnetic field strength for enhanced sensitivity and resolution.[42] Unlike MS and UV, NMR provides direct structural information

that furnishes structural elucidation (Table 2).[74] From a set of 1D spectra, it is possible to get information on the qualitative (^{1}H or ^{13}C) and quantitative (signals intensities) composition of the investigated structure as well as the identity of functional groups (chemical shift) and bond properties (*J* couplings). Unlike MS and UV, NMR is highly non-selective, which means that it can detect compounds with no chromophore or with poor ionization in MS.[75] For dereplication purposes, NMR detection can be obtained on-line either through direct hyphenation (on-flow, stopped-flow, or loop-storage), through a solid-phase extraction (SPE) interface,[76,77] or it can be used as an off-line detection method.

LC-NMR has been used extensively for the dereplication of plant-derived metabolites.[75,78] However, there are only few examples of this method used for the dereplication of marine[72,79] or microbial extracts.[80-82] Crews' lab has used NMR-based dereplication for the characterization of diphenyl ethers and sesterterpenes in sponge extracts.[83,84] Likewise, Dias and Urban (2008) used a combination of on-line and off-line LC-NMR for the dereplication of halogenated monoterpenes from a marine algae, *Plocamium mertensii*.[85] To the best of my knowledge, the only example of NMR-based dereplication of a marine bacterium is from the cyanobacterium, *Ficherella ambigua*.[86] Here, Lin et al. (2008) applied off-line microdroplet NMR in order to identify novel antibacterial ambiguines in an extract consisting of mainly known compounds.

The key strategy used for dereplication in the Marine Chemistry Group at the University of Canterbury (UoC) consists of a combination of HPLC-UV, and off-line MS and capillary NMR (CapNMR),[87] well integrated with their biological screening system.[81] The eluate from a single HPLC run is collected in a 96-well microtiter plate, and wells are combined to provide single-peak material for direct injection MS and CapNMR. The first level of dereplication is based on simple 1D ^{1}H-NMR data that usually is acquired in 5-10 minutes (for MW < 600 Da).[87] The spectrum is inspected for NMR recognizable features, such as the number of methyl groups and their multiplicity, or coupling patterns of substituted benzene rings. These features can in a numerical way be used as query in AntiMarin to perform very powerful and discriminatory searches. Often a DQF-COSY spectrum can be recorded in a reasonable amount of time that in some cases can provide a substructure that can be used as query in the various databases, incl. SciFinder where NMR data and accurate mass cannot be used.

During my stay at UoC, I had the chance to use this approach for the dereplication of an extract of *Pseudoalteromonas luteoviolacea*. Antibacterial activity of the extract was suspected to be ascribed to the dual presence of violacein and indolmycin (**paper 1**) (more details in section 3.3.2). Indolmycin had only been isolated from terrestrial actinomycetes,[88,89] thus in order to validate its presence in *Pseudoalteromonas*, a pure fraction suspected to contain indolmycin was analyzed by CapNMR. Initial inspection of the 1D spectrum revealed the presence of two methyl groups, one doublet and one singlet, the latter possibly an N-methyl based on the chemical shift value of δ_H 2.8 ppm. In addition, the characteristic splitting pattern of a tryptophan unit was seen (Figure 3.8). Feeding this set of information from the 1D ^{1}H-NMR spectrum to AntiMarin together with the nominal mass 257 Da, resulted in a single candidate: Indolmycin. A full NMR dataset of pure indolmycin corroborated this result. The use of CapNMR for dereplication enabled the absolute identification of a known compound rather than just tentative as can be obtained by LC-UV/MS.

NMR still trails MS in terms of the minimum sample amount required.[77] From the experience at UoC, only 200-500 µg of initial extract is needed to provide sufficient material for the NMR analysis, usually giving 2-50 µg of pure compound.[87] We found this amount to be insufficient for the extract of *P. luteoviolacea*. In order to obtain sufficient amounts of pure compound, multiple HPLC runs were necessary, and due to the complexity of the extract manual fraction collection was required in order to ensure adequate purity. Like for any nanomole or micromole NMR technique, the purity of the peaks is of outmost importance as the signals are not easily deconvoluted at this scale. This means that the slightest impurities can hamper any interpretation of the spectra.[42,77] In consequence, one has to be very careful in construction of a database query. A wrong input can lead to too many restrictions, and thus a false negative. Many other setups for NMR-based dereplication exist; however, the mentioned limitations (Table 2) are valid for all.

LC-NMR dereplication can in the best case scenario completely obviate any large-scale purification.[86,87] Occasionally, it is possible to identify novel compounds directly from a crude extract, thus condensing dereplication, purification, and structure elucidation into a single step.[87,90] However, for most *de novo* structure determinations it is necessary to do large scale purification in order to supply sufficient material for a full dataset and determination of the absolute configuration of a compound by X-ray crystallography.

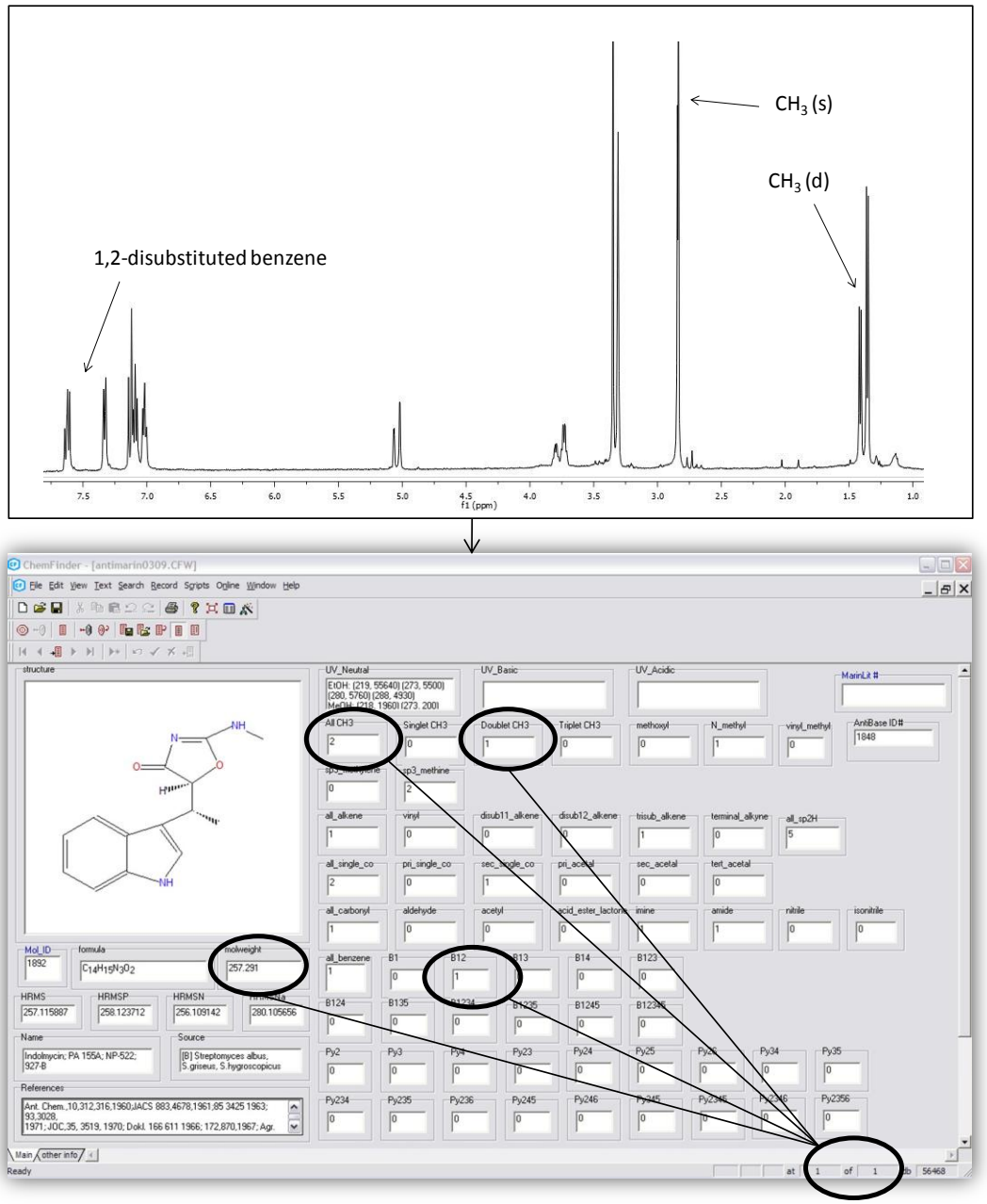


Figure 3.8 Dereplication of a pure fraction from *Pseudoalteromonas luteoviolacea* strain S4054 using CapNMR and AntiMarin 2009. Top: 1D ^{1}H-NMR spectrum of indolmycin recorded in MeOD (reference, δ_H 3.31 ppm) using CapNMR (32 scans, 1 min 30 sec). The sample contains a mixture diastereomers. Bottom: AntiMarin database query with nominal mass MW 257 and NMR recognizable features, i.e. two methyls including one doublet methyl and a 1,2-disubstituted benzene. The query provides one potential candidate with these restrictions: Indolmycin.

	LC-UV/MS	CapNMR
Advantages	10^{-12}-10^{-20} mole Automated acquisition No collection necessary Deconvolution possible Suitable for complex mix Suitable for many databases	Non-destructive 10^{-6}-10^{-9} mole Non-selective Positive ID of knowns Information unambiguous
Limitations	Destructive Tentative ID of knowns Not very discriminatory Poorly ionizing compounds	Manual acquisition No deconvolution Not suitable for complex mix Collections necessary Few databases

Table 2 Comparison of principal advantages and limitations of MS and NMR-based dereplication methods.

3.3.2 Explorative Solid-Phase Extraction (E-SPE) for accelerated dereplication of complex extracts

During the work with marine bacterial metabolites, we found that both the approach at CMB and UoC to be insufficient due to the convoluted nature of the samples. LC-UV/MS was not sufficiently discriminatory and could not provide the necessary answers of whether to pursue with purification of a given extract, while the CapNMR approach was too insensitive and time-consuming for complex extracts. In order to contribute to additional facets to the dereplication process, we have developed the so-called explorative solid-phase extraction method (**paper 1**).

The intent was to construct a setup based on solid-phase extraction (SPE) columns that could provide essential information on functional groups present in an extract. Such information is suitable as query in available commercial databases like AntiBase. Based on statistical analysis of all metabolites reported in AntiBase 2008 and their theoretical pK_a values, we found that charge is a very discriminatory factor for the dereplication of microbial metabolites (Figure 3.9).

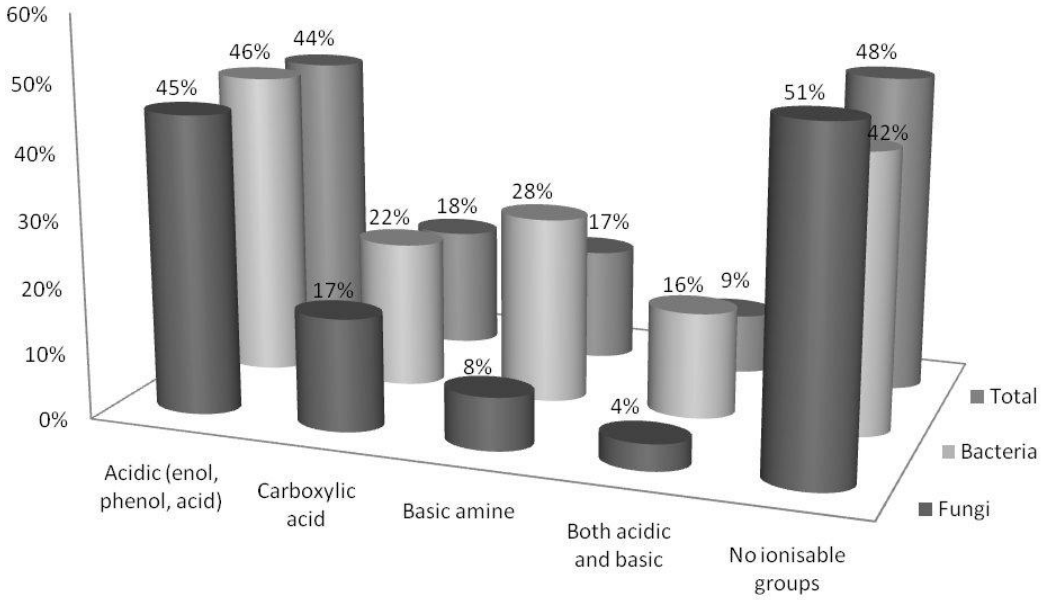


Figure 3.9 Distribution of microbial natural products in AntiBase 2008 with charged functionalities within pH range 2-11, excluding permanently charged groups such as sulphates, phosphates, and tertiary amines as well as potential tautomeric forms (**paper 1**).

Some 44% of all compounds were found to have an acidic functionality, either represented as a carboxylic acid (18%) or as an acidic phenol or enol. In consequence, we decided to include two anion-exchangers, i.e. a strong anion-exchanger (SAX) for retention of carboxylic acids and a mixed-mode anion-exchanger (MAX) for additional retention of weak acids. Owe to the dual selectivity of the MAX column, information of the relative polarity of the extract components could also be obtained. The number of compounds with a basic amine functionality added up to 17%, leaving a positive charge as the most discriminatory for dereplication purposes. So, we decided to include a strong cation-exchanger (SCX) in the setup. Interestingly, there was a markedly higher fraction of basic functionalities in bacterial compounds (28%) compared to fungal (8%). Also, more compounds had an amphoteric nature (16%). This is possibly a reflection of different biosynthetic pathways in bacteria and fungi, with a higher frequency of alkaloids, non-ribosomal peptides or hybrids hereof in at least in certain groups of bacteria (**chapter 4**).[91,92] Thus, cation-exchange for the dereplication and purification of bacterial metabolites is very useful.

As a fourth column, we decided to include Sephadex LH-20 for size-exclusion. In natural product chemistry there is a strong tradition for the use of LH-20 for orthogonal purification to reversed-phase C_{18}. LH-20 is a very versatile column material with dual hydrophilic and hydrophobic properties that separates compounds on the basis of molecular size and shape. The material shows low irreversible absorption, giving high sample recovery, and various solvent interactions and hydrogen bonding result in good affinity towards phenolics and heteroaromatic compounds.[93] The combination of the SAX, MAX, SCX, and LH-20 columns provides orthogonal information of charge, polarity, and size of extract components to be used in dereplication and purification.

For some extracts, we found a diol column to be an attractive alternative to the use of LH-20. Essentially, diol is a normal-phase column and will separate based on polarity like C_{18}. However, the hydroxyl functionalized backbone provides excellent resolution for charged compounds,[94] and in many cases we found diol be orthogonal to C_{18} (Figure 3.10) rather than pure silica where opposite elution (and thus not orthogonal) is often seen. Other polar-functionalized bonded silicas such as cyano- and aminopropyl were considered for the E-SPE setup. Bonded-phase silicas show good mass recovery and have weak ion-exchange properties when used with aqueous solutions. These columns can be used with advantage for a second round of optimizations.

The specific conditions were optimized for the individual columns, including loading, solvents and additives, dimensions, and number of fractions. Based on AntiBase 2008 statistics, we found most compounds with an ionizable functionality to be charged at pH 2 and 11 for anions and cations, respectively (**paper 1**). Thus, these values were used for sample load and elution on the ion-exchangers. The strong anion- and cation-exchangers were run in a simple 'on-off' mode, yielding two fractions per column with unretained (A1/C1) and retained (A2/C2) compounds, respectively (Figure 3.11, page 56). To avoid surpassing the capacity of the strong ion-exchangers, careful desalting of marine extracts is very important. The dual selectivity of the MAX column was exploited by the collection of six fractions corresponding to polar (B1), medium-polar (B2), and apolar (B3) neutral and basic compounds and polar (B4), medium-polar (B5), and apolar (B6) acidic compounds. Several gradients were attempted; however, the best resolution, as defined by presence of activity in a single fraction, was obtained with 25, 60, and 100% organic.

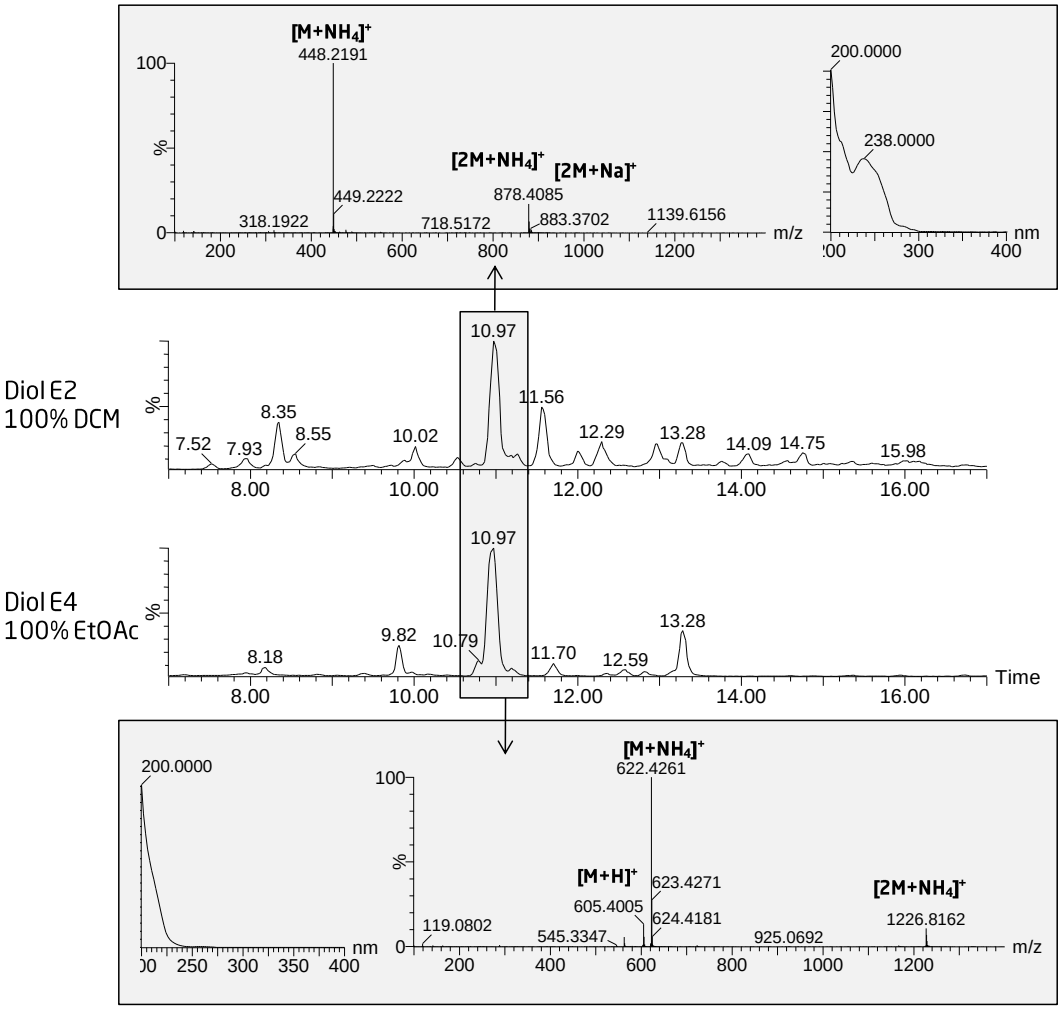


Figure 3.10 Comparison of LC-MS chromatograms of E-SPE fractions E2 and E4 (*Photobacterium halotolerans* s2753) from diol, corresponding to 100% dichlormethane (DCM) and 100% ethylacetate (EtOAc). Both fractions contain a peak with Rt = 10.97 min on a reversed-phase C_{18} column, however with distinct masses and UV spectra.

The best resolution for LH-20 was obtained through band-based collection rather than time-based (D1-D5). However, this type of collection is restricted to colored extracts and makes it difficult to run columns on different extracts in parallel. No pre-packed columns with LH-20 exist, thus a certain degree of variation was introduced for this column due to manual packing. Different column dimensions were tried, but we found no significant differences between columns at this small scale (<100 mg dry material). We found the most important factor for good resolution on LH-20 to be the loading conditions. Extracts should be loaded in as small a volume as possible for optimal focusing on the column. Also, the presence of a frit in top of the column improved resolution. When diol was included in the setup, six fractions were collected corresponding to 100% heptane (E1), 100% dichlormethane (DCM) (E2), 50/50 v/v DCM/ethylacetate (EtOAc) (E3), 100% EtOAc (E4), 50/50 v/v EtOAc/methanol (MeOH) (E5), and 100% MeOH (E6).

Based on the SAX, MAX, SCX, LH-20 setup, at total of 15 fractions was obtained (Figure 3.11, 16 fractions if using diol). We decided to run the four SPE columns in parallel as opposed to in series. Even though a sequence of SCX, followed by SAX and MAX would yield 13 fractions rather than 15, information about the stability of compounds on the individual columns would be lost.

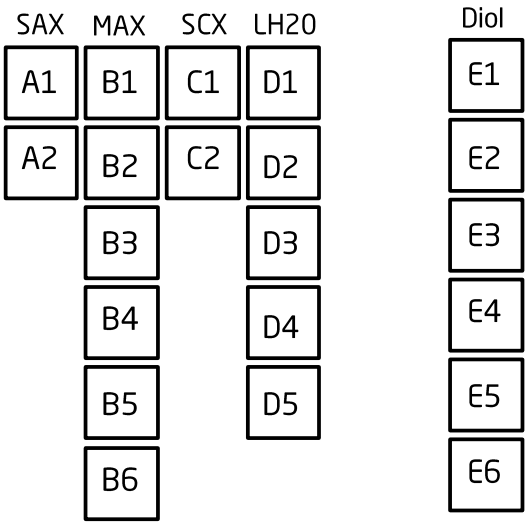


Figure 3.11 Fractions collected during E-SPE. From the SAX column: A1 = unretained neutrals and bases, A2 = retained carboxylic acids. From MAX: B1, B2, B3 = polar (25% MeOH), medium-polar (60% MeOH), and polar (100% MeOH) neutrals and bases, B4, B5, B6 = polar (25% MeOH), medium-polar (60% MeOH), and polar (100% MeOH) acids including phenols and enols. From SCX: C1 = unretained neutrals and acids, C2 = retained bases. From LH20: D1-D5 bands originating from compounds of decreasing size. From diol: 100% heptane (E1), 100% DCM (E2), 50%/50% DCM/EtOAc (E3), 100% EtOAc (E4), 50%/50% EtOAc/MeOH (E5), and 100% MeOH (E6).

Linked to a bioassay system, the power of E-SPE for dereplication is enhanced. Minor constituents of an extract can exhibit exceptional potency in a given bioassay, and thus the sensitivity of our assays was exploited to extract chemical information. By comparison of LC-UV/MS traces of active and non-active fractions, it was possible to identify peaks as potential candidates for an observed bioactivity (comparative dereplication). The orthogonality of the E-SPE setup makes it possible to assess the number of bioactive constituents in an extract, and the contribution from individual compounds could be estimated by accounting from the titer.

To ensure broad applicability of E-SPE, we did various tests to ensure the compatibility with all our bioassays. As the target organisms in most of our assays were sensitive to acid, it was necessary to use formic or acetic acid to regulate pH on the ion-exchangers as both would evaporate off more quickly than the solvent. For marine extracts, efficient desalting was imperative as formate and acetate ions formed under acidic conditions caused interference in growth inhibition assays. Also, components such as bile acids (originating from yeast extract) in many complex media were found cause interference at increased concentrations. Thus, in order to be able to compare the assay response, we found it imperative to include a blank medium sample and preferable only use simple media for cultivation.

In order to validate the E-SPE method, we used several extracts of *Pseudoalteromonas* as they are known to be prolific in their production of bioactive natural products (**paper 3**).[51,92] The E-SPE bioactivity profiles of four strains of *P. luteoviolacea* against *Staphylococcus aureus* revealed two distinct phenotypes (Figure 3.12). Type 1, representing strains S2607 and S4060, produced at least two antibacterial compounds, one being apolar with an amine and acidic phenol or enol (observed in fractions A1, B6, C2, and D5), and the other being medium to apolar with an acidic phenol or enol (A1, B5, C1, and D2). These functionalities were consistent with the presence of violacein and pentabromopseudilin (PL1, see section 3.2.2), and the presence of both was confirmed by LC-UV/MS (**paper 3**).

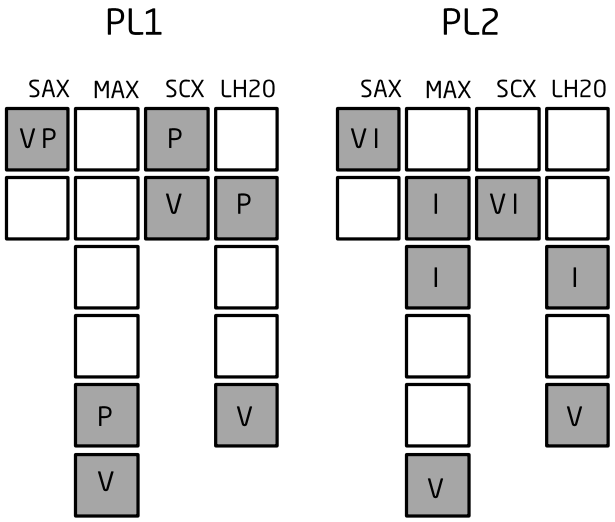
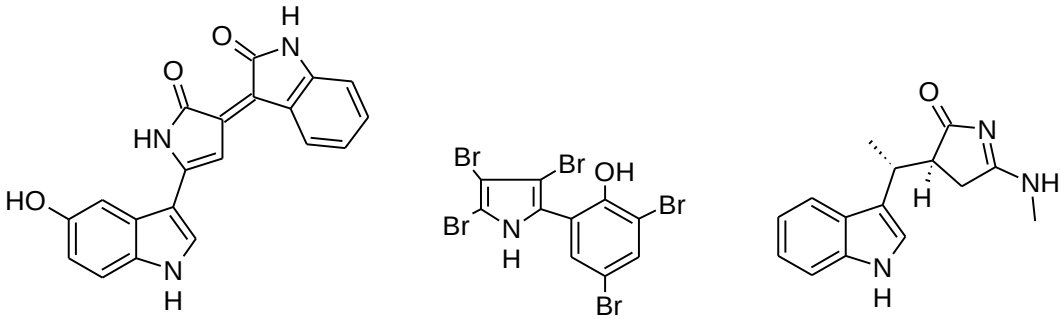


Figure 3.12 E-SPE bioactivity profiles (growth inhibition of *Staphylococcus aureus*) of chemotypes PL1 and PL2 of *Pseudoalteromonas luteoviolacea*. Grey boxes denote growth inhibition. The presence of violacein (V), pentabromopseudilin (P), and indolmycin (I) as confirmed by LC-UV/MS is marked on the individual fractions



Violacein **Pentabromopseudilin** **Indolmycin**

E-SPE type 2, representing strains S4047 and S4054 (PL2, see section 3.2.2), had a bioactivity pattern corresponding to violacein and as well as an unknown antibacterial compound also with a basic functionality (A1, B2-B3, C2, and D3). This compound was also active against *Vibrio anguillarum* (**paper 1**). Further interest in this compound was fueled by activity of the E-SPE fractions in QSI selector assays based on *lux* and *las/rhl* QS systems (Figure 3.13),[95] an activity that was undetected in the crude extract of *P. luteoviolacea* (unpublished results). As the *las/rhl* QS system control the expression of various virulence factors in *Pseudomonas aeruginosa*, the unknown compound has potential anti-pathogenic activity.[96]

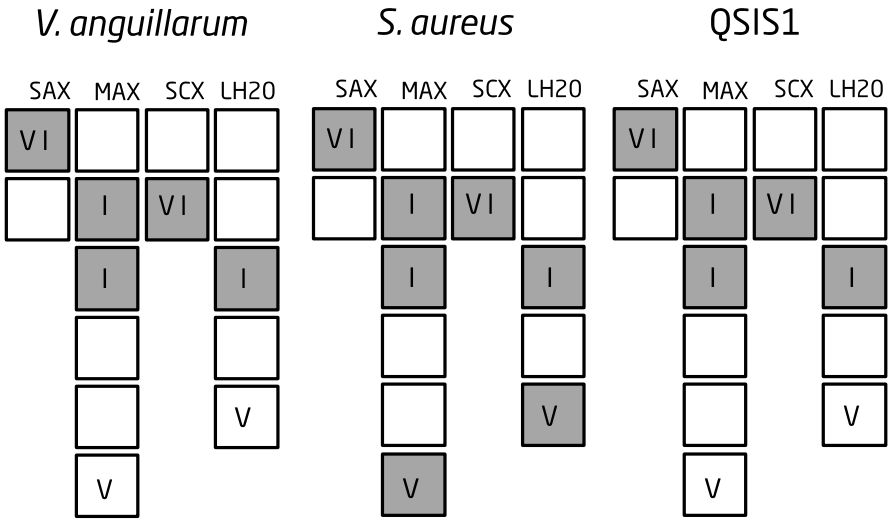


Figure 3.13 E-SPE bioactivity profiles of chemotype PL2 of *P. luteoviolacea* in growth inhibition assays against *Vibrio anguillarum*, *Staphylococcus aureus*, and in the QSI selector 1 based on *Vibrio fischeri luxR*. Grey boxes denote inhibition. The presence of violacein (V) and indolmycin (I) as confirmed by LC-UV/MS is marked on the individual fractions.

Comparative dereplication by LC-UV/MS led to the tentative identification of indolmycin (**paper 1**). From the E-SPE results it was possible to design a purification strategy based on cation-exchange and size-exclusion for rapid, target-guided isolation of indolmycin. The identity of the pure compound was verified by NMR (section 3.3.1) and biological testing confirmed that indolmycin was in fact responsible for the observed activity. Re-testing of pure indolmycin at various concentrations revealed that the ability to down-regulate virulence gene expression was only valid in a very narrow concentration window. At higher concentrations, indolmycin was strongly inhibitory against *Pseudomonas aeruginosa* (Holm Jakobsen, unpublished results). This example demonstrates the power of E-SPE for mapping of multiple bioactivities in a complex extract, unmasking subtle bioactivities by pre-fractionation prior to biological testing, accelerated dereplication, and target-guided purification based on targeted exploitation of ionizable functionalities.

Targeted exploitation of chemical functionalities can shortcut the dereplication and purification of an active compound. Yet, only 52% of the metabolites in AntiBase have a charged functionality. In the extract of *Photobacterium halotolerans* S2753, the active components were unretained on all ion-exchangers. Nonetheless, by comparison of the bioactivity profile in the *Staphylococcus aureus* growth and quorum sensing inhibition assays, it was evident that the antibacterial compound, holomycin (**paper 4**) and the QS inhibitors, solonamide A and B (**paper 6**) could easily be separated by the use of a diol column (Figure 3.14). This example demonstrates how E-SPE and pre-fractionation based on orthogonal SPE columns is suitable for secondary screening and planning purification strategies for extracts containing active compounds with no charged functionalities.

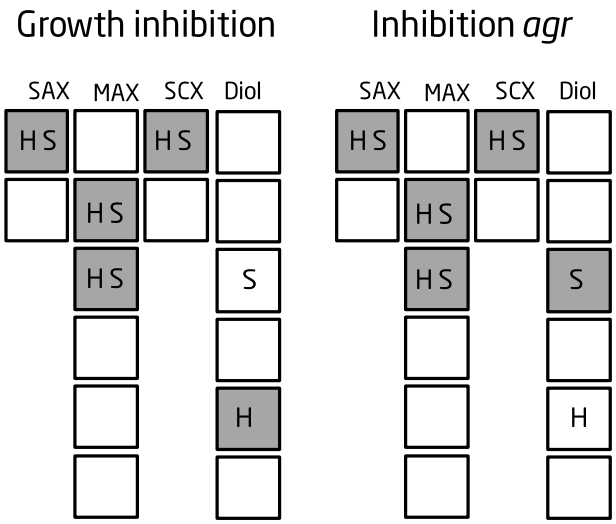
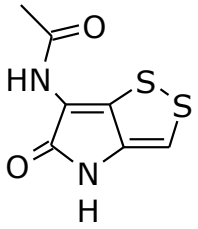
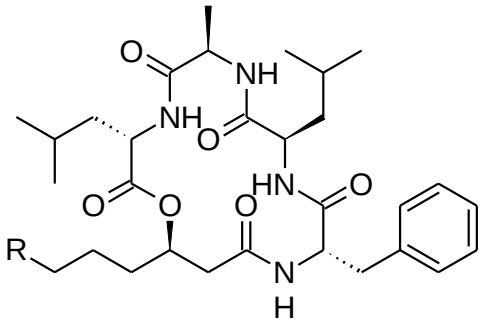


Figure 3.14 E-SPE bioactivity profiles of *Photobacterium halotolerans* strain s2753 in a *S. aureus* growth inhibition assay and an assay monitoring inhibition of quorum-sensing (*agr)* regulated virulence in *S. aureus.* The bioactivity profiles show the presence of a least two active compounds: the antibacterial compound, holomycin (H) present in fractions A1, B1-B2, C1, and E5, and the antivirulence compounds, solonamide A-B (S) are present in A1, B2-B3, C1, and E3.



Holomycin (H)



Solonamides (S)
Solonamide A, R = H
Solonamide B, R = Et

3.4 Profiling and dereplication of marine bacteria (conclusions)

Natural products represent an unparalleled pool of chemical diversity with complex biological activities that lead to competitive ecological advantage for the producing organism. This makes natural products an attractive resource for drug discovery. Today, more than 230,000 natural products are reported,[97] and every year more than 1000 new structures are added only from microorganisms.[98] That stresses the need for new and improved screening and dereplication methods that reduce the risk of structural redundancy to keep natural products as a viable option in drug discovery programs.

Front line chemical evaluation of crude extracts at CMB employs LC-UV/MS metabolite profiling of bacteria displaying interesting biological activities. Chemical profiling is an efficient tool to characterize a bacterium based on its biosynthetic potential and makes it possible to rank extracts according to a criterion of novelty. We found the use of chemical profiling by LC-UV/MS very useful for marine bacteria such as *Vibrio* and *Pseudoalteromonas* (**paper 3-6**). It enabled the grouping of similar strains at species and sub-species level disregarding geographical sampling locations, thus countering the perception that secondary metabolites in bacteria are strictly strain-specific. Nonetheless, intra-species variations demonstrate why metabolite profiling should be an integral part of an intelligent screening strategy in order to uncover the full biosynthetic potential of a given species.

More detailed dereplication can be obtained after pre-fractionation. For simple extracts, on-line and off-line NMR-based methods can be used to augment the data obtained from LC-MS, providing absolute identification of known compounds on the expense of small amounts of extract. We found NMR-based methods to fall short for the dereplication of complex bacterial extracts due to lack of sensitivity. As a complement, we developed explorative solid-phase extraction (E-SPE) (**paper 1**) that uses charged functionalities as discriminatory features. Orthogonal fractionation of an extract yields simpler chromatograms and allows comparative dereplication of active and non-active fractions, which can highlight potential candidates for observed biological activity and unmask compounds with more subtle bioactivities such as quorum sensing inhibition. Also, E-SPE showed that different chemotypes in *Pseudoalteromonas luteoviolacea* (**paper 3**) were linked to different bioactivity profiles, and accelerated the isolation of the key metabolites responsible for the differentiation (**paper 1**). E-SPE is an example of a new dereplication method that is well

integrated with other steps of the discovery process such as purification and structure elucidation.

Dereplication needs to be performed at various stages of the discovery process, and should not be seen as a single process but rather a continuous critical evaluation for prioritization of extracts, fractions, and compounds. Based on the instruments and analytical facilities available it is possible to make a 'dereplication decision tree', like the one seen in Figure 3.15 (page 65), to support the risk/probability assessment of the active compound(s) being known or novel. The strategy being pursued will depend on the compound classes present, the overall complexity of the extract, and biological activities being targeted.

Based on the methods used during this PhD thesis and experiences gained, I suggest the following setup for the dereplication of microbial extracts (refer to decision tree in Figure 3.15):

- LC-UV/MS is used to provide a rapid overview of the compounds in a bioactive extract:

    - The chromatogram is dominated by compounds with PKS/NRPS-like spectral features (highly conjugated systems with characteristic UV spectra) or compounds with poor ionization → Assessment of the complexity of the extract: Is the chromatogram dominated by few peaks with good base-line separation on a RP C_{18} column? **YES/NO**

    - The chromatogram is dominated by compounds with peptide-like spectral features (good ionization, no chromophore) → LC-MS/MS: Is it possible to obtain a *de novo* sequence for structural elucidation? **YES/NO**

- Dereplication by NMR for simple extracts (few peaks, good base-line separation): Scale-up to 1 L culture → Absolute identification of pure compound (for some compounds also in mixtures), **known/novel?**

- Dereplication by E-SPE for complex extracts (many compounds, multiple biological activities, small span in polarity of extract components): Scale-up to 1 L culture → Tentative identification based on comparative dereplication and ionizable functionalities, **known/novel?**

- Assessment of the biological activity: Does the active fraction have activity towards a new or interesting biological target? **YES/NO**

- Large-scale purification and full structural elucidation performed only on dereplicated compounds. If the active principle is a known compound, it might be favorable to synthesize the compound (especially for peptides and simple structures) or a commercial standard may be available for purchase.

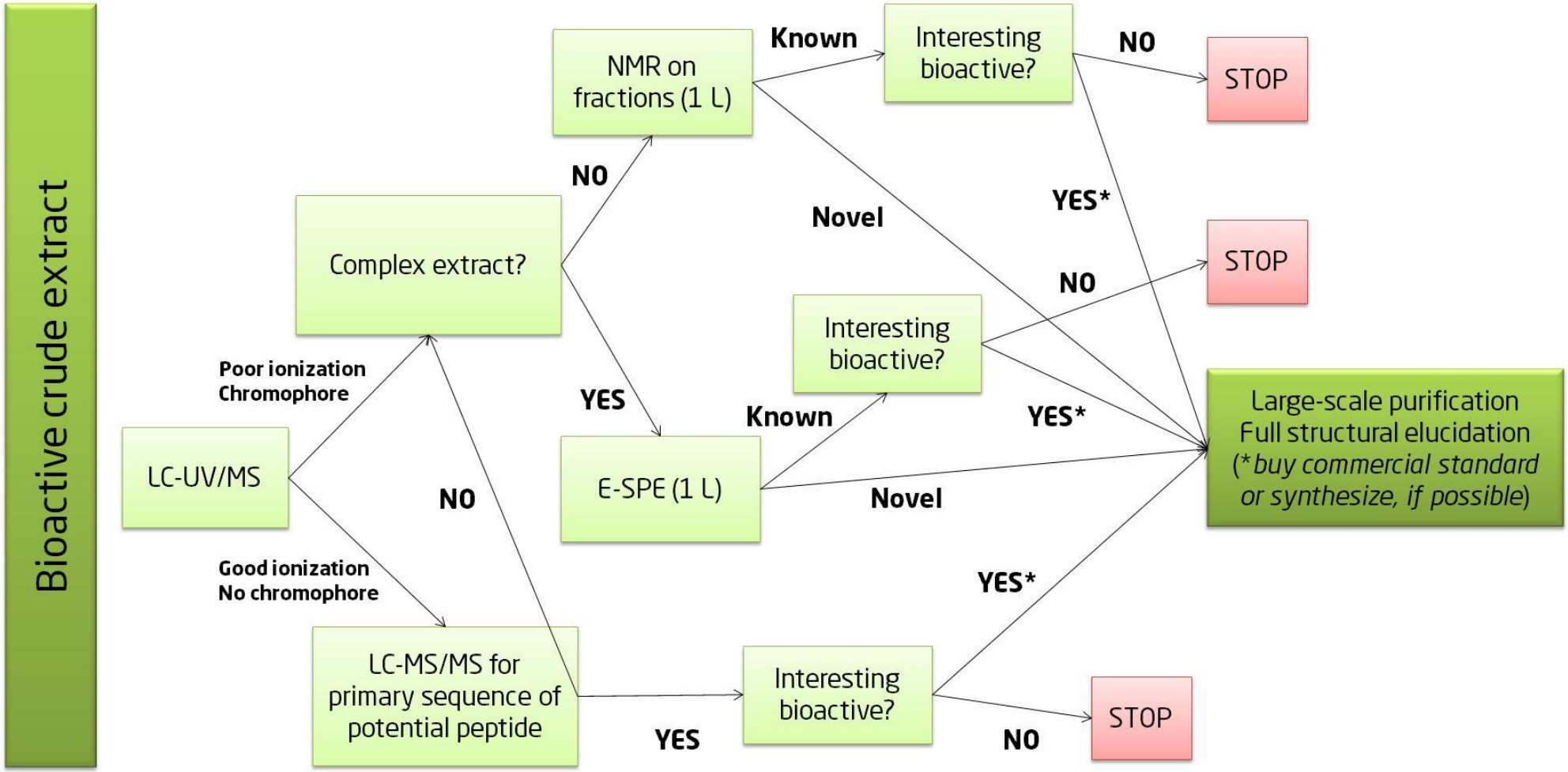


Figure 3.15 Possible dereplication 'decision-tree' based on the methods applied in this PhD study. Refer to the text for details.

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4. Marine *Vibrionaceae*

Marine *Vibrionaceae* are Gram-negative, rod-shaped γ-proteobacteria that are usually motile and possess a chemoheterotrophic metabolism.[1] Members of this family are widespread in the marine environment, including estuaries, coastal waters, and sediments.[1] The family includes eight genera (Figure 4.1): *Allivibrio* (5 species), *Enterovibrio* (4 species), *Salinivibrio* (5 species), *Catenococcus* (1 species), *Grimontia* (1 species), *Listonella* (2 species), *Vibrio* (81 species), and *Photobacterium* (20 species).[1,2]

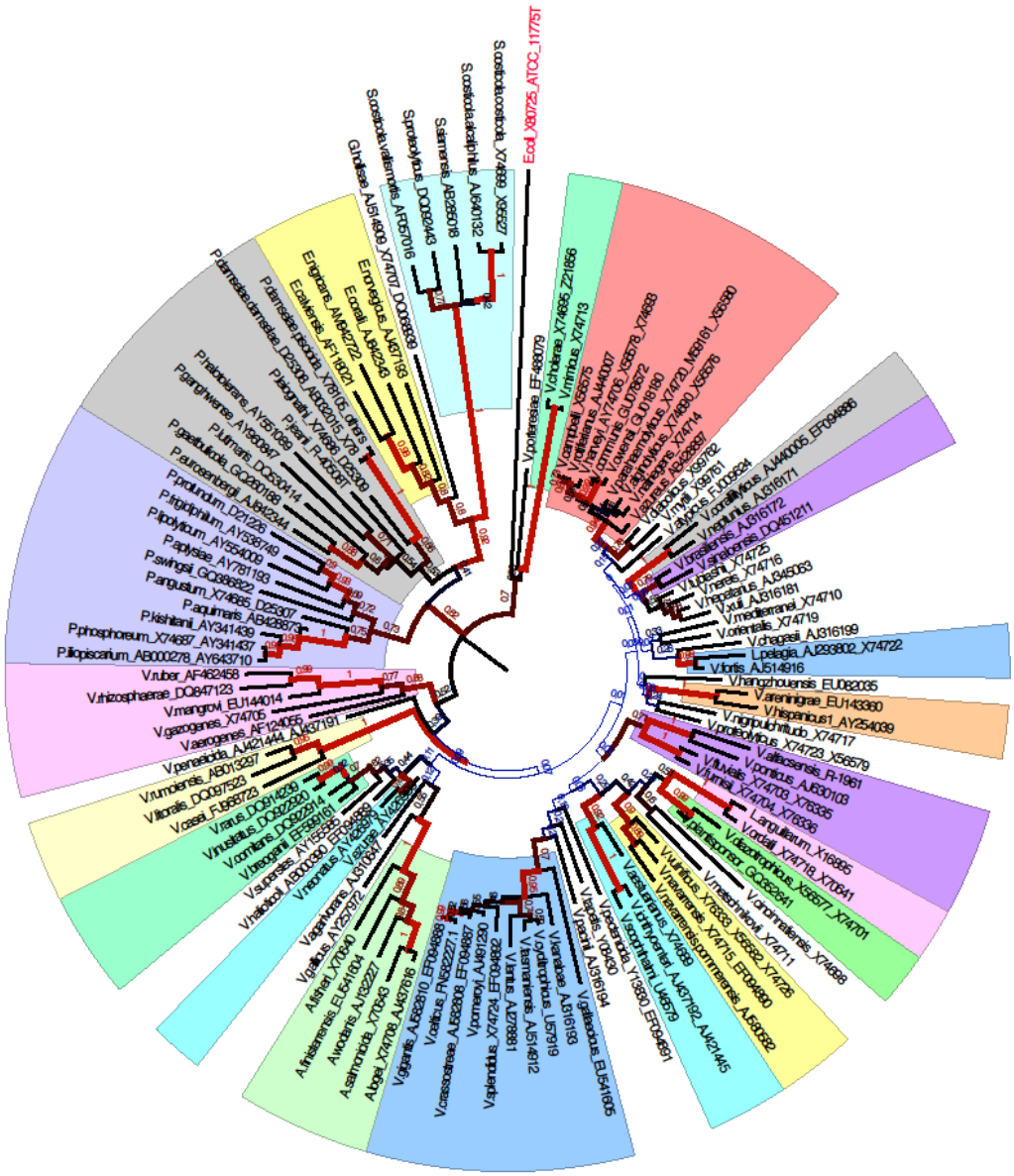


Figure 4.1 Phylogenetic tree of the *Vibrionaceae* family based on 16S rRNA gene sequence similarities (Association of *Vibrio* biologists).[2]

4.1 Occurrence and ecological significance

Vibrios are particularly abundant on the surface of marine macroorganisms such as corals, fish, seagrass, sponges, and zooplankton, where they form commensal, symbiotic, and pathogenic associations.[1] *Vibrio fischeri* and its symbiotic host, the Hawaiian squid *Euprymna scolopes*, is the model system for studying light organ symbioses.[3] *V. fischeri* colonizes the squid light organ and provides bioluminescence for the squid to use as countershading in order to evade predators. In return, the bacteria gain a protected nutrient environment. The symbiosis is highly specific. Studies have shown that even closely related bacteria like *V. parahaemolyticus*, or even *V. fischeri* from another niche, are not symbiosis-competent.[4] Besides *V. fischeri*,[3] *P. leiognathi* and *V. logei*[5] are known to exist in symbiotic relationships with squid, while *P. leiognathi* and *P. phosphoreum* can be associated with fish.[6]

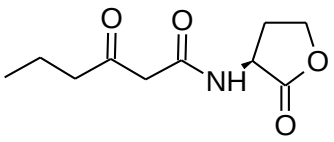
The *Vibrionaceae* comprise strains being opportunistic pathogens of humans and marine animals. *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* are serious human pathogens. *V. cholerae* probably has the greatest impact on human health, causing the acute diarrheal disease cholera that can result in epidemics.[7] It is a very persistent bacterium that can survive on a variety of vectors, including zooplankton[8] and cyanobacteria[9]. *V. parahaemolyticus*[10] and *V. vulnificus*[11] are food-borne pathogens associated with the ingestion of raw seafood. *V. vulnificus* is the more virulent of the two and causes primary septicaemia with high mortality via a battery of virulence factors, including hemolysins and proteases, siderophores, and capsular and endotoxic polysaccharides.[11] *V. anguillarum*, *V. salmonicida*, and *V. vulnificus* are important fish pathogens and are widespread in aquaculture settings, where conditions seem to enhance their virulence.[1] Vibrios are indigenous to healthy coral microbiomes; however, of nine coral infectious diseases, six are caused by members of this family.[12] Increased seawater temperature can lead to a proliferation of opportunistic pathogens like *V. coralliilyticus* or *V. shilonii* (*V. mediterranei*) that can cause coral bleaching.[1] Also, *P. rosenbergii* and *Enterovibrio coralii*[13] have been suspected to be involved in coral disease. In *V. coralliilyticus*, virulence has been linked to enhanced levels of a zinc metalloprotease that can cause bleaching and coral tissue lesions.[12]

High densities of *Vibrio* and *Photobacterium* on the surface of zooplankton[14] have in part been ascribed to the ability of vibrios to utilize chitin, an N-acetyl D-glucosamine polymer in zooplankton exoskeletons, as carbon and nitrogen source.[15] The presence of chitinases and chitinase encoding genes has been confirmed for several members of the family.[15-17] Chitin has been found to

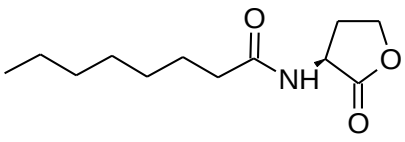
control several genetic and physiological characteristics of vibrios[18] including antagonistic activity (**paper 5**). Also, vibrios are able to degrade various other complex sugars such as fucoidan and laminarin found in various algal species (**paper 5**).[19] Thus, this superior nutrient utilization might be one of the reasons for the ubiquitous presence of vibrios in the marine environment.[20]

The ability to form biofilms is widespread among vibrios, playing a significant role in the pathogenicity of *V. cholerae,*[21] *V. parahaemolyticus,* and *V. vulnificus,*[22] as well as in the symbiotic colonization by *V. fischeri.*[23,24] Key proteins include pili, lectins, exopolysaccharides, and components involved in the formation of flagella.[22] Though vibrios share a high number of regulatory systems of biofilm formation, there are differences that could reflect different niche specificity or ecological roles.[25] For example, it appears that vibrio produce species-specific exopolysaccharides, the major component of bacterial biofilms, and often have the potential to produce more than one type.[22]

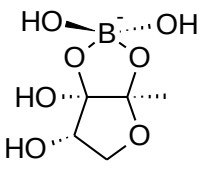
Vibrios use quorum sensing (QS) to control traits such as luminescence, virulence, and biofilm formation. The discovery of *N*-acylhomoserine lactones as QS signals was first made in *V. fischeri* and *V. harveyi*, and the LuxI/R system of *V. fischeri* is the paradigm of Gram-negative quorum sensing systems even though it is not found in all vibrios.[26] In *V. fischeri*, there are three distinct QS signals; 3-oxo-C6-HSL (**1**), C8-HSL (**2**), and AI-2 (**3**). These are used to control a regulatory cascade leading to induction of luminescence. The existence of multiple QS systems demonstrate the ability of vibrios to respond to variable marine conditions.[26]



(1) *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL)



(2) *N*-octanoyl-L-homoserine lactone (C8-HSL)



(3) AI-2

4.2 Genomic diversity and phylogeny

In contrast to most γ-proteobacteria, vibrios possess two circular chromosomes.[27,28] Essential functions and housekeeping genes are usually located on the large chromosome ChrI, which is rather constant in size (~3 Mb), while the smaller ChrII is flexible in size, ranging between 0.8-2.4 Mb.[29] ChrII contains accessory genes related to transcriptional regulation, for example, pathogenicity and antimicrobial resistance.[29,30] Genes encoding for chitin metabolism and quorum sensing are split between the two chromosomes.[29] Val and Mazel (2009) showed that fusing the two chromosomes[31] yielded healthy and viable cells. However, generation times were markedly longer, and vibrios with only one chromosome were easily outcompeted by the wild-type. The ability of vibrios to vary the copy numbers of the two chromosomes is suspected to be involved in the adaptation to varying environmental conditions.[30] Horizontal gene transfer is involved in the genetic flexibility of vibrios, including transduction by phages, plasmid conjugation[32], and so-called 'super-integrons'.[33,34] In addition, Meibom et al. (2005) showed that vibrios become naturally competent when grown in the presence of chitin,[35] allowing uptake of free DNA from the environment. Chitin-induced competence has been demonstrated in *V. cholerae,*[35] *V. vulnificus,*[36] and *V. fischeri.*[37] Altogether, this indicates vibrios being well-suited for rapid acquisition of niche-specific functions.

The high genomic diversity of vibrios can be directly translated into high phenotypic variability.[28] This makes it difficult to obtain meaningful groupings of vibrios at genus and species level based on isolated phenotypical markers.[1] Also, the 16S rRNA gene is highly conserved among the *Vibrionaceae* and not well suited for identification to the species level.[38] Attempts to improve the taxonomy include sequencing and comparison of various housekeeping genes, including *recA*, *rpoA*, *toxR*, which hold greater sequence variability than 16S.[38-41] Taxonomy of vibrios by genetic markers has been supplemented by chemical analyses, including fatty acid methyl ester (FAME) profiling, and more recently by whole-cell MALDI-TOF MS,[42,43] and LC-UV/MS chemical profiling (**paper 4, 5**). Chemotyping was found to be especially useful at sub-species level, identifying differences in antibiotic production (**paper 5**). Whole-cell MALDI-TOF MS was able to distinguish closely related species like *V. parahaemolyticus* and *V. alginolyticus* or *V. cholerae* and *V. mimicus* scouting potential biomarkers within a 4,000-14,000 Da mass range.[42] Closely related species (*V. coralliilyticus* and *V. neptunius*) could be distinguished based on their secondary metabolite production (**paper 4**).

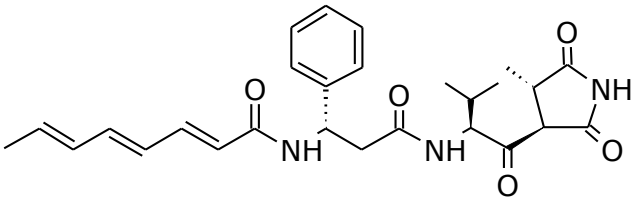
4.3 Natural product production by members of the *Vibrionaceae* family

Considering their widespread presence in the marine environment, vibrios are largely underexplored for their proclivity to produce secondary metabolites. From *Vibrionaceae*, a total of 91 compounds have been reported in literature. The majority of these compounds have been described in only three species; *V. parahaemolyticus*, *V. anguillarum*, and *V. vulnificus*, which is likely a reflection of their importance as pathogens. Based on their average genome size,[29,44] the vibrios are expected to be less abundant in their production of secondary metabolites as compared to, for example, marine actinomycetes.[45] In the following, all metabolites reported from *Vibrionaceae* (Table 1) will be presented and interesting compounds highlighted in an attempt to assess the chemical diversity and biosynthetic potential of this group of bacteria.

4.3.1 Compounds with antibacterial activity

Marine vibrios are known producers of antibiotics in the marine environment,[46-48] where these compounds potentially contribute to their abundance in surface-associated communities.[49] Long and Azam (2001) studied antagonistic interactions among pelagic bacteria and found that vibrios produced broad-range antibacterial compounds.[50] Similar capabilities of vibrios have been observed with coral-associated bacteria.[51] The relatively widespread production of antibiotics in marine vibrios (**paper 4**) indicates that antagonistic activity may be of ecological importance (**paper 5**).

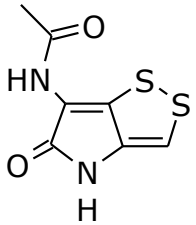
Probably the best studied antibiotic produced by vibrios is the hybrid NRPS-PKS peptide antibiotic andrimid (**4**).[52] The compound interferes with fatty acid biosynthesis[53] and is effective against a wide range of bacteria.[54] Structure-activity studies by Pohlmann et al. (2005) revealed the pyrrolidinedione head was essential for activity, while variations in the fatty acid tail was more tolerable.[53] This suggested that these two structural moieties play different roles in target binding.



(4) Andrimid

Andrimid is a cosmopolitan antibiotic found in distantly related bacteria, including a symbiotic *Enterobacter* sp. from the planthopper *Nilaparvata lugens,*[55] *Pseudomonas flourescens,*[56] *Pantoea agglomerans,*[57] and vibrio.[52] The andrimid biosynthetic gene cluster was found to be conserved in two different producers.[58] Interestingly, the cluster encodes resistance genes[59] as well as specific transposases that could be responsible for the diverse occurrence of this antibiotic.[57,58] From vibrios, the compound was first isolated by Oclarit et al. in 1994,[52] and Long et al. (2005) identified andrimid as the compound responsible for the growth inhibition of *V. cholerae* by an unidentified *Vibrio* strain.[48] We isolated andrimid from the culture broth of a *V. corallillyticus* strain (**paper 4**) isolated from sediment in the tropical Indian Ocean,[60] thus for the first time linking production of andrimid to a specific vibrio species. The production of andrimid was found to be part of different chemotypes within *V. corallilyticus* (**paper 5**). Two Galathea-strains, S2052 and S4053 from two distant geographical locations, were found to produce the antibiotic (**paper 4**), while the type strain and a close relative did not produce it. Interestingly, *V. coralliilyticus* S2052 focused its production of secondary metabolites to the production of andrimid when grown on chitin, which mimicked natural growth conditions, at the same time increasing the yield of the antibiotic (**paper 5**). This indicated that andrimid potentially contributes to different niche-specificities of *V. coralliilyticus*.

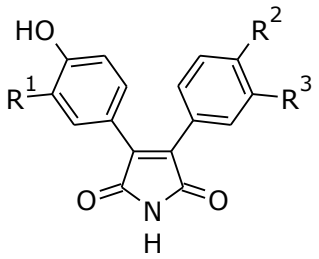
We isolated the highly potent pyrrothine antibiotic, holomycin (**5**) from *Photobacterium halotolerans* strain S2753 that originated from a mussel collected in the tropical Pacific (**paper 4**). Prior to this isolation, holomycin had only been isolated from actinomycetes, including *Streptomyces clavuligerus,*[61] *S. griseus,*[62] and a marine *Streptomyces* sp.[63]



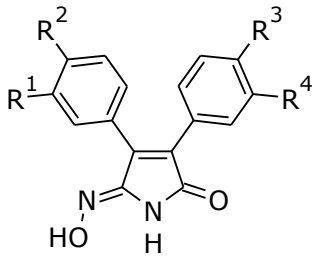
(5) Holomycin

The NRPS biosynthetic cluster encoding holomycin in *S. clavuligerus* was recently identified by Li and Walsh (2010),[64] and this allows for the comparison the holomycin clusters in other producers, including *Photobacterium*. Holomycin has a broad spectrum of antibacterial activity against pathogenic bacteria such as *Staphylococcus aureus*, *S. pneumoniae*, *S. epidermis*, *Enterococcus faecalis*, and *Escherichia coli*.[65] The mode-of-action in *E. coli* was found to include inhibition of RNA chain elongation, but holomycin is suspected to act as prodrug rather than a direct inhibitor of the RNA polymerase.[65] We found holomycin to be strongly inhibitory against several marine strains from *Roseobacter*, *Pseudoalteromonas*, and *Vibrio*, including pathogens like *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* (**paper 4**). This suggested that holomycin might play a role in antagonism in the marine environment.

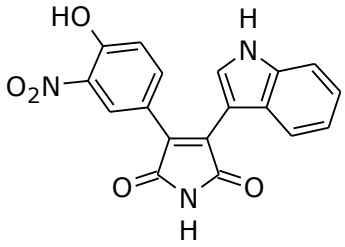
Yao and Al-Zereini recently (2010) isolated a series of nitrosubstrituted maleimides called aqabamycins (**6-14**) from a coral-associated *Vibrio* sp.[66,67] The analogues had varying antibacterial activity against Gram-positive bacteria, including *Micrococcus luteus*, *Bacillus subtilis*, and *B. brevis* as well as cytotoxic activity.[67] The aqabamycins represent a unique structural group both due to their high degree of nitrosubstitution which is rare in nature[68] and the maleimide monoxime present in aqabamycin E (**10**-**11**) and F (**12**).



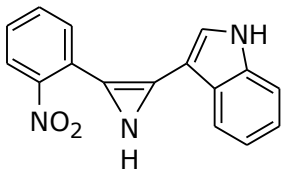
(6) Aqabamycin A, R^1 = H , R^2 = H , R^3 = H
(7) Aqabamycin B, R^1 = H , R^2 = OH , R^3 = NO_2
(8) Aqabamycin C, R^1 = NO_2 , R^2 = H , R^3 = H
(9) Aqabamycin D, R^1 = NO_2 , R^2 = OH , R^3 = NO_2



(10) Aqabamycin E, R^1 = NO_2 , R^2 = OH , R^3 = H , R^4 = H
(11) Aqabamycin E', R^1 = H , R^2 = H , R^3 = OH , R^4 = NO_2
(12) Aqabamycin F, R^1 = NO_2 , R^2 = OH , R^3 = OH , R^4 = NO_2



(13) Aqabamycin G



(14) Aqabamycin H

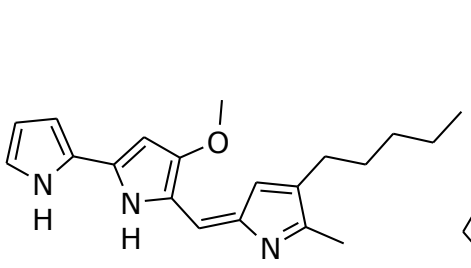
Table 1 Compounds reported from marine *Vibrionaceae* and their associated biological activities:

	Name	Compound class	Source	Other activities	References
Antibacterial	**Andrimid (4)**	Pyrrolidinedione	*V. coralliilyticus*		Oclarit 1994[52] Wietz 2010 (**paper 4**)
	Aqabamycin A (6)	Nitro maleimide	*Vibrio sp.*	Cytotoxic	Yao 2010[66]
	Aqabamycin B (7)				
	Aqabamycin C (8)				
	Aqabamycin D (9)				
	Aqabamycin E (10)	Maleimide oxime			
	Aqabamycin E' (11)				
	Aqabamycin F (12)				
	Aqabamycin G (13)	Nitro maleimide			
	Aqabamycin H (14)	Azirine			Yao 2007[69]
	B-4607-C	Phenazine	*Vibrio sp.*		Sato 1995[70]
	Cycloprodigiosin (16)	Prodiginine	*V. gazogenes*		Gerber 1983[71]
	3,5-Dibromo-2-(3',5'-dibromo-2'-methoxyphenoxy)-phenol	Diphenyl ether	*Vibrio sp.*	Antifungal	Elyakov 1991[72] Sionov 2005[73]
	2,2-Di-(3-indolyl)-3-indolone	Indole	*V. parahaemolyticus*		Bell 1994[74] Veluri 2003[75]
	Griseoluteic acid	Phenazine	*Vibrio sp.*		Sato 1995[70]
	Holomycin (5)	Pyrrothine	*P. halotolerans*		Wietz 2010 (**paper 4**)
	Indazole-3-carbaldehyde	Indazole	*Vibrio sp.*	Anticancer	Yao 2010[66]
	Magnesidin A (17)	Tetramic acid Mg^{2+} salt	*V. gazogenes*	Antialgal	Imamura 1994[76]
	Moiramide B	Pyrrolidinedione	*Vibrio sp.*		AntiBase 2010[77]
	Ngercheumicin I (20)	Depsipetide	*Photobacterium sp.*		Adachi 2007[78]
	Ngercheumicin II (21)				
	Pelagiomicin C	Phenazine	*Vibrio sp.*	Anticancer	Sato 1995[70] Imamura 1997[79] Singh 1997[80]
	Prodigiosin (15)	Prodiginine	*V. psychroerythrus* *V. gazogenes* *V. ruber*	Antiprotozoan, antifungal, anticancer	D'Aoust 1974[81] Harwood 1978[82] Shieh 2003[83]
	Turbomycin	Indole	*Vibrio sp.* *(V. parahaemolyticus)*	Antifungal	Veluri 2003[75] Yao 2007[69]
	Unnarmicin A (18)	Depsipeptide	*Photobacterium sp.*	Antifungal	Oku 2008[84]
	Unnarmicin C (19)				
	Vibrindole A	Indole	*V. parahaemolyticus*	Antifungal	Bell 1994[74] Yao 2007[69]
Siderophore	**Anguibactin (27)**	Catechol hydroxamate	*V. anguillarum*	Cytotoxic	Jalal 1989[85] Sandy 2010[86]
	Aerobactin	Hydroxamate	*Vibrio sp.*		Haygood 1993[87]
	Amphibactin B	Hydroxamate (amphiphilic)	*Vibrio sp.*		Martinez 2003[88]
	Amphibactin C				
	Amphibactin D				
	Amphibactin E				
	Amphibactin F				
	Amphibactin G				
	Amphibactin H				
	Amphibactin I				
	Bis-[3-(2,3-dihydroxy-benzoylamino)-propyl]-amin	Catechol	*V. fluvialis*		Yamamoto 1993[89]
	Bisucaberin (32)	Hydroxamate	*V. salmonicida*	Anticancer	Takahashi

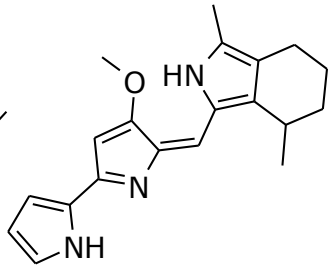
					1987[90] Winkelmann 2002[91]
	Divanchrobactin	Catechol	*Vibrio sp.*		Sandy 2010[86]
	Fluvibactin (31)	Catechol Hydroxyphenyl-oxazolone	*V. fluvialis*		Yamamoto 1993[89]
	Trivanchrobactin	Catechol	*Vibrio sp.*		Sandy 2010[86]
	Vanchrobactin (28)	Catechol	*V. anguillarum*		Soengas 2006[92]
	Vibriobactin (20)	Catechol Hydroxyphenyl-oxazolone	*V. cholerae*		Griffiths 1984[93]
	Vibrioferrin	Carboxylate	*V. parahaemolyticus*		Yamamoto 1994[94]
	Vulnibactin (29)	Catechol Hydroxyphenyl-oxazolone	*V. vulnificus*		Okujo 1994[95]
	Vulnibactin 2	Vulnibactin precursor			
	Vulnibactin 3				
Anticancer	**Kahalalide F (33)**	Depsipeptide	*V. mediterranei (V. shilonii)*	Antibacterial, antimalarial, antifungal	Hill 2005[96]
	Kahalalide H				Hill 2005[96]
	Kahalalide J				
Quorum sensing interference	**AI-2 (3)**	Furanosyl borate diester	*Vibrio*	QS	Chen 2002[97]
	***N*-hexanoyl-L-homoserine lactone**	Homoserine lactone	*V. anguillarum*	QS	Kuo 1994[98]
	***N*-(3-hydroxybutanoyl)-L-homoserine lactone**	Homoserine lactone	*V. harveyi*	QS	Cao 1989[99]
	***N*-(3-hydroxyhexanoyl)-L-homoserine lactone**	Homoserine lactone	*V. anguillarum*	QS	Milton 2001[100]
	[1-(2'-methylpropoxy)-2-hydroxy-2-methyl-propoxy]butane (41)		*P. angustum (V. angustum)*	QS	De Nys 2001[101]
	***N*-(3-oxodecanoyl)-L-homoserine lactone**	Homoserine lactone	*V. anguillarum*	QS	Milton 1997[102]
	***N*-(3-oxohexanoyl)-L-homoserine lactone (1)**	Homoserine lactone	*V. fischeri* *V. cholerae* *V. harveyi* *V. anguillarum*	QS	Eberhard 1981[103] Milton 2006[26]
	***N*-octanoyl-L-homoserine lactone (2)**	Homoserine lactone	*V. fischeri*	QS	Kuo 1994[98]
	Solonamide A (35)	Depsipeptide	*P. halotolerans*	QSI Gram pos	Månsson 2011 (**paper 6**)
	Solonamide B (36)				
Na channel blocker	**Anhydro-tetrodotoxin**		*Vibrio sp.*		Noguchi 1987[104] Lee 2000[105]
	4-epi-tetrodotoxin		*Vibrio sp.*		Noguchi 1987[104] Lee 2000[105]
	Tetrodonic acid		*Vibrio sp.*		Noguchi 1991[106] Lee 2000[105]
	Tetrodotoxin (34)		*V. harveyi* *V. alginolyticus* *V.fischeri*		Noguchi 1986[107] Noguchi 1987[104] Lee 2000[105]
Riboflavin synthase inhibitor	**7-hydroxy-6-methyl-8-(1-D-ribityl)lumazine**	Pteridine	*P. phosphoreum*		Suzuki 1973[108]
	Photolumazine A				
	Photolumazine B				
	Photolumazine C				
Misc.	**Arundine**	Indole	*V. parahaemolyticus*		Veluri 2003[75]
	Benzoic acid	Aromatic	*Vibrio sp.*		Yao 2010[66]
	3,3-Bis-(3-	Indole	*V. parahaemolyticus*		Veluri 2003[75]

indolyl)butan-2-one			
3,3'-Bisindolylmethane			
1,4-dithiane		*Vibrio sp.*	Yao 2010[66]
3-hydroxybenzoic acid	Aromatic	*Vibrio sp.*	Yao 2010[66]
4-hydroxycinnamic acid			
p-Hydroxyphenyl-acetamide	Aromatic	*V. parahaemolyticus*	Veluri 2003[75]
Indole-3-carboxaldehyde	Indole	*V. parahaemolyticus*	Bell 1994[74]
Indole-3-acetic acid	Indole	*Vibrio sp.*	Gutierrez 2009[109]
6-methyl-8-D-ribityl-2,4,7-trioxopteridine	Pteridine	*P. phosphoreum*	Matsuura 1973[110]
3-nitro-4-hydroxy-benzaldehyde	Nitro aromatic	*Vibrio sp.*	Yao 2010[66]
3-nitro-4-hydroxycinnamic acid			
3-nitro-1*H*-indazole			
Pharacine (47)	Terephthalic ester	*V. parahaemolyticus*	Veluri 2003[75]
Phenylacetic acid	Aromatic	*Vibrio sp.*	Yao 2010[66]
Phenyl-2-*bis*-indolylmethane	Indol		
Photopterin A	Pteridine	*P. phosphoreum*	Matsuura 1973[110]
8-D-ribityl-2,4,7-trioxopteridine			
Trisindoline	Indole	*V. parahaemolyticus*	Veluri 2003[75]
1,1,3-Tris-(3-indolyl)butane			
1,1',1''-Trisindolyl-methane (46)			

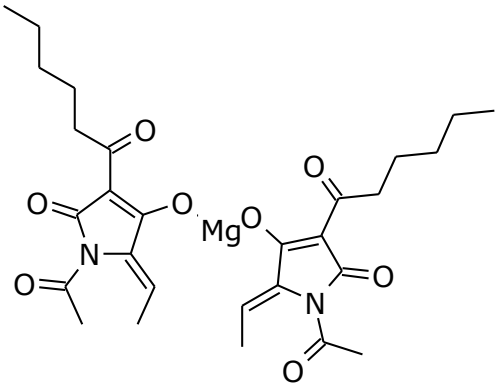
Table 1 Compounds reported from marine *Vibrionaceae* and their associated biological activities**.** Excluded from the list are sugars, fatty acids, and small peptides commonly found in marine culturable bacteria. Excluded are also compounds from AntiBase 2010 whose presence could not be confirmed in any reference regarding *Vibrionaceae*.



(15) Prodigiosin



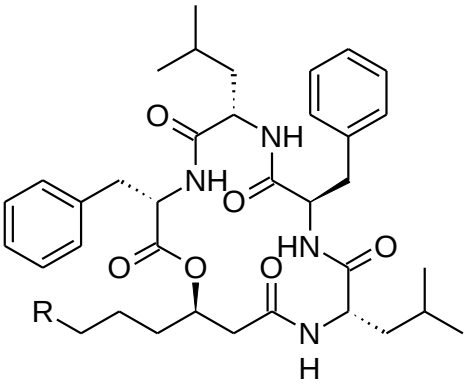
(16) Cycloprodigiosin



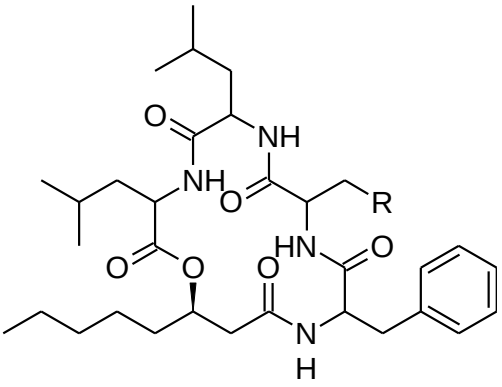
(17) Magnesidin A

The red pigment and antibiotic prodigiosin (**15**) has been isolated from *V. psychroerythreus,*[81] *V. gazogenes* (originally termed *Beneckea gazogenes* but later revised),[82] and *V. ruber*.[83] Additional producers of this compound include *Alteromonas rubra*/*Pseudoalteromonas rubra* (**paper 3**),[111] *Hahella chejuensis,*[112] and various *Serratia*[113] and *Streptomyces*.[114,115] Prodigiosin and its cyclized analogue (**16**)[71,116] have a broad range of biological activities, including antimicrobial, antimalarial, immunosuppressive, and anticancer.[117-119] Prodiginines have clinical potential in anticancer therapy,[119] and prodigiosin is currently in preclinical trials (Aida Pharmaceuticals) for pancreatic cancer.[117] The clinical potential as antibiotics, however, is limited due to a low therapeutic window and considerable toxic effects.[120] Starič et al. (2010) recently demonstrated that the production of prodiginines in a *Vibrio* sp. isolated from estuaries conferred competitiveness against a *Bacillus* sp. from the same sample,[121] suggesting that prodigiosin might act as a antibiotic in the natural environment. Interestingly, the prodigiosin producing *V. gazogenes* was also found to produce the unique magnesium containing antibiotic, magnesidin (**17**).[76,122-124]

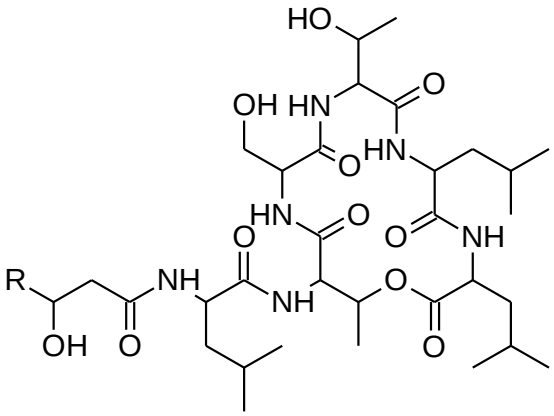
Shizuri and co-workers isolated two distinct groups of depsipeptides, the unnarmicins[84] and ngercheumicins[78] from a *Photobacterium* sp. with potent, but narrow-spectrum antibacterial effect against strains of *Pseudovibrio*. The unnarmicin A (**18**) and C (**19**) consist of four amino acids (L-Phe, L-Leu, D-Phe, L-Leu) and a 3-hydroxyoctanoic and 3-hydroxyhexanoic fatty acid, respectively. The ngercheumicins have a depsipeptide macrocycle and either a fatty acid (type I) (**20**) or peptide tail (type II) (**21**). They have been patented for treating infections caused by *Pseudovibrio denitrificans*, though no literature describes pathogenic traits of this bacterium.[125] We isolated five different type II ngecheumicins (**22-26**) from cultures of the Galathea strain S2753 closely related to *Photobacterium halotolerans* (Kjærulff and Månsson, unpublished data).[126] As no general nomenclature concerning the ngercheumicins exists at this writing, these new compounds are designated ngercheumicin A-E. Ngercheumicin A and B have a fully saturated tail, while C, D, and E contain a single double bond. The potential antibacterial effect of these compounds is yet to be determined.



(18) Unnarmicin A, R = Et
(19) Unnarmicin C, R = H



(20) Ngercheumicin 'I'



(21) Ngercheumicin 'II'
(22) Ngercheumicin A, R = $C_{11}H_{23}$
(23) Ngercheumicin B, R = $C_{13}H_{27}$
(24) Ngercheumicin C, R = C_9H_{17}
(25) Ngercheumicin D, R = $C_{11}H_{21}$
(26) Ngercheumicin E, R = $C_{13}H_{25}$

4.3.2 Siderophores

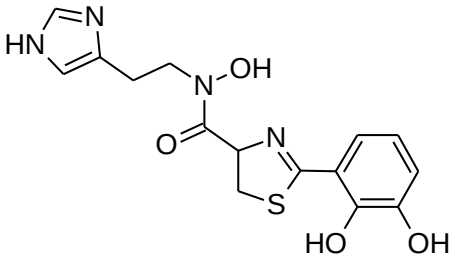
Many vibrios produce siderophores as a strategy to sequester iron in the marine environment, where the iron level is extremely low.[127,128] This is necessary to maintain important enzymatic processes (with iron as cofactor) and a prerequisite for pathogenicity for many vibrios.

A great structural diversity has been observed among the siderophores produced by *Vibrio* species. In *V. anguillarum*, at least two different siderophore-mediated systems have been described, namely anguibactin (**27**)[85,129] and vanchrobactin (**28**).[130] The non-ribosomal peptide anguibactin represents a unique structural class of siderophores with both a catechol and hydroxamate ligand and a thiazole core.[85] The biosynthetic genes encoding this compound are found on a 65-kb virulence plasmid in some *V. anguillarum* strains. Knock-out of genes involved in anguibactin production was found to attenuate virulence, confirming that anguibactin is a prerequisite for successful host-invasion of this bacterium.[131] In contrast, the catechol vanchrobactin is chromosome-encoded, and interestingly, the coding genes are silenced in anguibactin producing strains.[92,132] Recently, dimeric and trimeric versions of vanchrobactin were isolated from an unidentified *Vibrio* by Sandy et al. (2010).[86] Also, they found anguibactin to possess cytotoxic activities against P388 murine leukemia cells.[86]

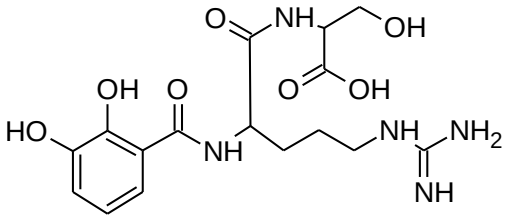
Vibriobactin (**30**),[93] vulnibactin (**29**),[95] and fluvibactin (**31**)[133] are unique siderophores produced by *V. cholerae*, *V. vulnificus*, and *V. fluvialis*, respectively. They are all catechol hydroxyphenyloxalone siderophores that share a rare norspermidine backbone, giving them a propeller-like structure. In vibriobactin and vulnibactin, two of the hydroxybenzoyl moieties are linked to the backbone through an L-threonine, forming an oxazoline ring. Fluvibactin only has one oxazoline ring, with one hydroxybenzoyl directly linked to the norspermidine terminal. Vibriobactin and vulnibactin differ only in the number of hydroxylations, and this high structural similarity enables cross-utilization of these two siderophores.[95]

Bisucaberin (**32**)[90] is a symmetric cyclic dihydroxamate produced by the fish pathogen *V. salmonicida*.[91] Unlike most other vibrio siderophores,[131] bisucaberin is produced through an NRPS-independent route[134] where alternating dicarboxylic acids and diamine or amino alcohols are assembled through amide or ester bonds.[135] Bisucaberin was found to be useful in combinatorial anticancer therapy by sensitizing tumor cells to macrophage-mediated cytolysis.[90,136]

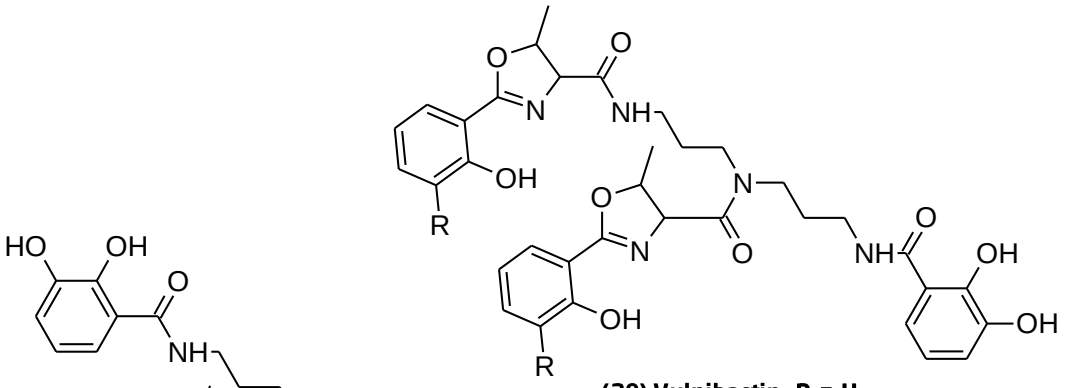
Siderophores are used as therapeutic deferration agents to treat iron overload in chronically transfused thalassemia patients. A stereochemically modified version of fluvibactin was found to efficiently remove iron without increasing microbial growth.[137] It has been suggested that siderophores can be used for the development of a new class of 'trojan horse' antibiotics.[138] Siderophore-antibiotic conjugates exploit the iron transport system of the pathogen to penetrate the bacterial outer membrane, increasing the antibacterial activity of the antibiotic.[139] Recently, Bergeron et al. (2009) made a conjugate linking antibodies to vulnibactin as a strategy towards a vaccine against *V. vulnificus*.[140]



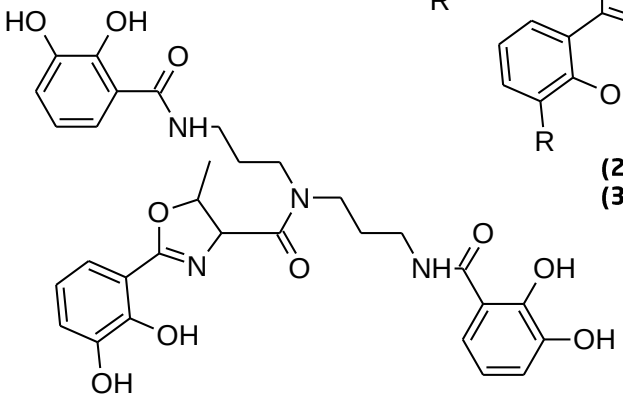
(27) Anguibactin



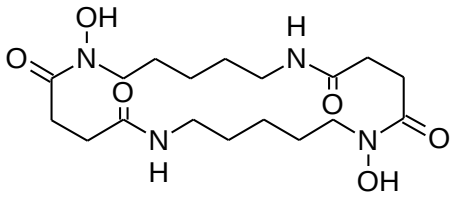
(28) Vanchrobactin



(29) Vulnibactin, R = H
(30) Vibriobactin, R = OH



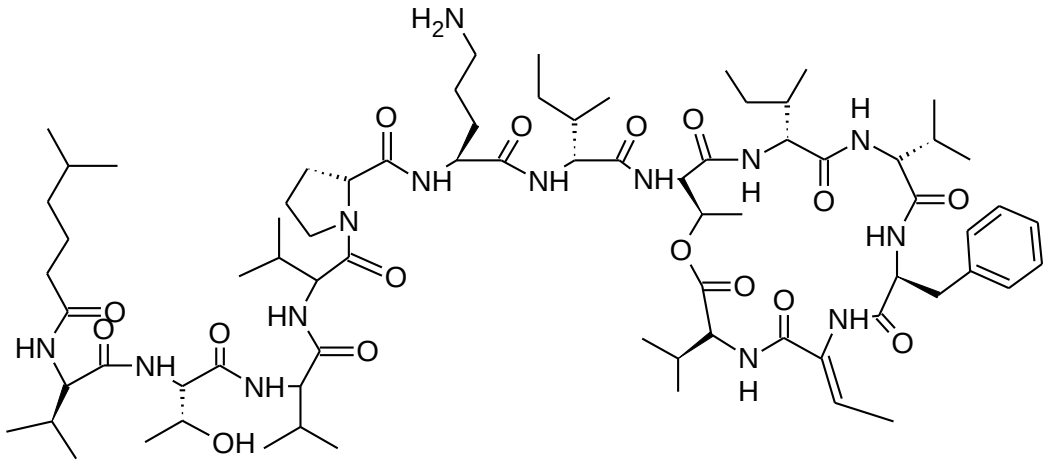
(31) Fluvibactin



(32) Bisucaberin

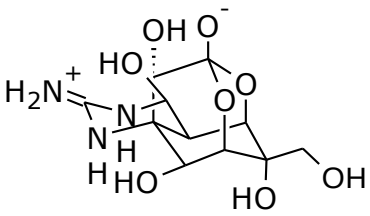
4.3.3 Compounds with other activities

Another interesting group of compounds produced by a member of the *Vibrionaceae* are the kahalalides. These cyclic depsipeptides were originally isolated from the herbivorous mollusc *Elysia refescens* and its diet, the green algae *Bryopsis* sp. In particular, kahalalide F (**33**) has an attractive spectrum of activities against AIDS-related opportunistic infections and various cancer cell lines.[141] Kahalalide F is currently undergoing Phase II clinical trials (PharmaMar) for the treatment of prostate, lung, and liver cancer[141] and in patients with severe psoriasis (PharmaMar/Marinomed).[142] Interestingly, Hill and Hamann (2005) found kahalalide F as well as two analogues to be produced by a *V. mediterranei/shilonii*.[96] The finding of a microbial origin for this compound allows for the large-scale industrial fermentation of this compound rather than arduous organic synthesis.



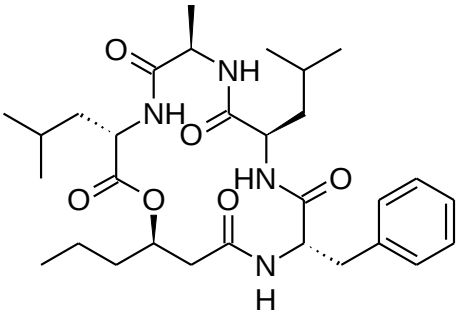
(33) Kahalalide F

Several vibrios produce the potent neurotoxin tetrodotoxin (TTX) (**34**), also known as the pufferfish poison.[105] The true origin of TTX has been the subject of much debate,[143] nonetheless *V. harveyi* and *V. alginolyticus* isolated from different species of pufferfish were found to produce the toxin as well as several analogues.[104] Also, *V. fischeri* isolated from the intestines of the xanthid crab, *Atergatis floridus* produced TTX.[107] The intestines of the pufferfish, *Fugu vermicularis vermicularis* were found to be dominated by vibrios,[104] suggesting that the toxification is caused by TTX-producing bacteria accumulated through the food web.[105] The role of these compounds to vibrio itself is still unclear, though it has been suggested to play a role in regulating sodium transport.[105]

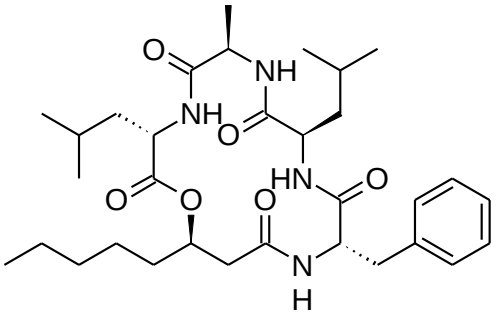


(34) Tetrodotoxin

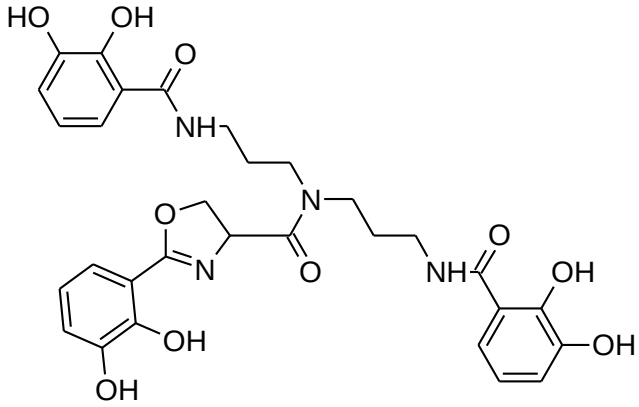
Vibrios produce compounds that interfere with the quorum sensing system of Gram-positive bacteria. From a *P. halotolerans* strain S2753 (**paper 4**), we isolated and identified two novel depsipeptides, solonamides A and B (**35-36**) that interfered with QS regulated virulence genes in *S. aureus* (**paper 6**). In particular, solonamide B dramatically reduced expression of both *hla* encoding α-hemolysin and *RNAIII*, while increasing expression of *spa* encoding Protein A. This suggested that the depsipeptides interfere with *agr*, the global virulence regulator in *S. aureus*. High structural similarity of the solonamides to the natural autoinducers of the *agr* system suggested that they might be competitive inhibitors. Interestingly, the solonamides were found to have a pronounced effect on virulence gene expression in *S. aureus* strain USA300, which is the predominant community-acquired MRSA (CA-MRSA) strain in the USA.[144] The solonamides strongly resemble the unnarmicins (**18-19**) found in an unidentified *Photobacterium* sp. (section 4.3.1).[84] Thus, it is possible that the unnarmicins also possess QSI activity. In addition to the solonamides, Nielsen and Phipps (manuscript in preparation) isolated nigribactin (**37**) from *V. nigripulchritudo* S2604 as inhibitor of the same system. Nigribactin belongs to the family of bactins that act as siderophores in *V. fluvialis* and others (section 4.3.2). The isolation of compounds belonging to very different structural classes as inhibitors of *agr* suggests that multiple modes-of-action may be employed. Thus, the elucidation of the mechanism for receptor binding is in the offing.



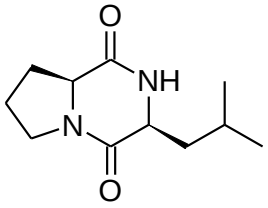
(35) Solonamide A



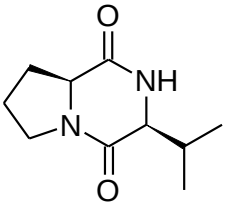
(36) Solonamide B



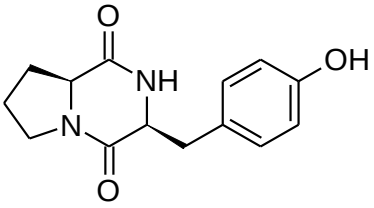
(37) Nigribactin



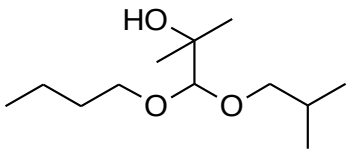
(38) *cyclo*(L-Pro-L-Leu)



(39) *cyclo*(L-Pro-L-Val)



(40) *cyclo*(L-Pro-L-Tyr)



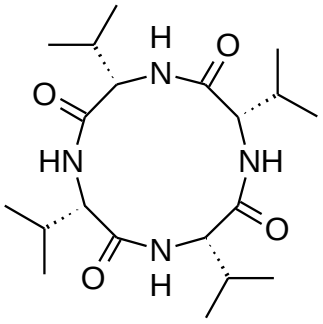
(41) [1-(2'-Methylpropoxy)-2-hydroxy-2-methylpropoxy]butane

Several small molecules isolated from vibrios have been found to induce Gram-negative quorum sensing systems. That includes various diketopiperazines (DKP). For example, *cyclo*(L-Pro, L-Leu) (**38**), *cyclo*(L-Pro, L-Val) (**39**), and *cyclo*(L-Pro, L-Tyr) (**40**), DKPs commonly isolated from vibrios,[74] were found to modulate LuxR-type protein activity though at higher concentrations than AHLs.[145] It is speculated that these dipeptides represent a new class of naturally occurring QS signals potentially involved in interspecies signaling, as DKPs are found in most culturable marine bacteria.[146] However, some DKPs are likely to be artifacts generated from media components during work-up procedures.[147] De Nys et al. (2001) isolated [1-(2'-methylpropoxy)-2-hydroxy-2-methylpropoxy]-butane (**41**) from *P. angustum* (*V. angustum*) S14 with the ability to mediate expression in two AHL-regulated systems, inducing bioluminescence in *V. harveyi* and the AHL reporter system in *Agrobacterium tumefaciens.*[101]

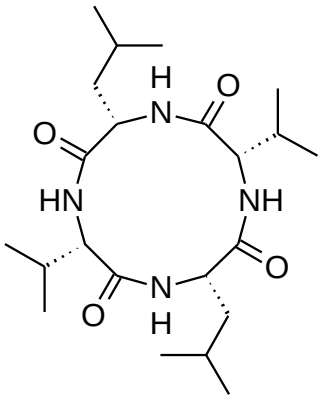
4.3.4 Compounds with unknown activities

Numerous compounds have been isolated from vibrios with no biological activity reported so far. That includes small-molecule by-products, for example some nitro-substituted compounds such as 3-nitroindazole and 3-nitro-4-hydroxycinnamic acid.[66] From *P. halotolerans* S2753, we isolated a series of cyclic tetrapeptides; *cyclo*(L-Val-L-Val-L-Val-L-Val) (**42**), *cyclo*(L-Val-L-Leu-L-Val-L-Leu) (**43**), *cyclo*(L-Val-L-Ile-L-Val-L-Ile) (**44**), and *cyclo*(L-Leu-L-Ile-L-Leu-L-Ile) (**45**) (Kjærulff and Månsson, unpublished data).[126] These types of peptides are often found in marine culturable bacteria,[148-151] suggesting that they are storage compounds accumulated during growth under excess nutrients. We did not find any of these peptides to have any antibacterial effects, which is in concordance with the literature on similar structures.

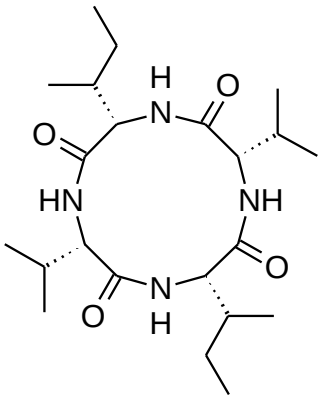
Many of compounds isolated from vibrios are suspected to be artifacts generated from media components during work-up procedures.[75,147,152] These include several bis- and trisindole derivatives from a *V. parahaemolyticus* strain, Bio249.[75] An example is 1,1,1-tris(3-indolyl)methane (**46**) that could easily be formed by simple condensation of indole-3-carbaldehyde and indole, both having been isolated from *V. parahaemolyticus.*[75] From the same *V. parahaemolyticus* strain, the cyclic terephthalic acid ester, pharacine (**47**)[152] was isolated.[75] This was suspected to be an artifact from plastic material contaminants; however, fermentation results were reproducible with no contact with plastic. Until biosynthetic studies have been performed, the true origin of these molecules remains uncertain.



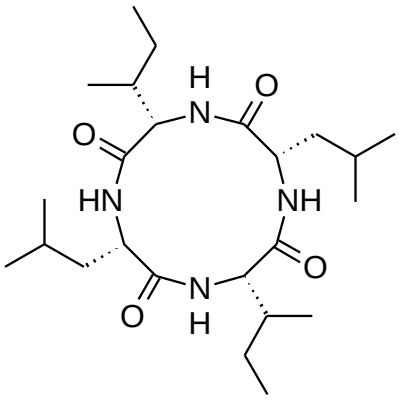
(42) *Cyclo* (L-Val-L-Val-L-Val-L-Val)



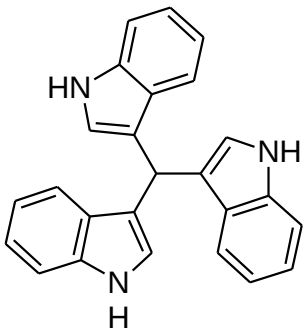
(43) *Cyclo* (L-Val-L-Leu-L-Val-L-Leu)



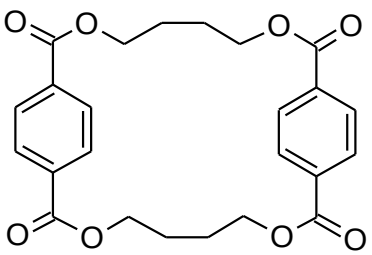
(44) *Cyclo* (L-Val-L-Ile-L-Val-L-Ile)



(45) *Cyclo* (L-Leu-L-Ile-L-Leu-L-Ile)



(46) 1,1,1-tris(3-indolyl)methane



(47) Pharacine

4.4 The biosynthetic potential (conclusions)

The versatility and widespread occurrence of vibrios can be ascribed to different characteristics; superior nutrient utilization, excellent biofilm formation, and their genetic construct. High genomic flexibility in *Vibrionaceae* makes this group of bacteria very apt to resist various environmental changes, for example through the acquisition of biosynthetic genes linked to the production of antibiotics or siderophores. So, rampant horizontal gene transfer occurs in these bacteria. As a reflection, most compounds isolated from vibrios have also been found in other types of bacteria, in many cases from distantly related taxa (**paper 4**). Sometimes the mobile genetic elements are even incorporated in the biosynthetic cluster itself, making it even more prone to gene-exchange. The antibiotic andrimid is an example of a compound encoded by such a 'nomadic gene cluster'.[153]

Production of secondary metabolites in vibrios has been linked to antagonism, intraspecies communication, and pathogenicity. The compounds produced by vibrios are mainly non-ribosomal peptides or hybrids hereof, with examples of N-containing compounds produced by NRPS-independent pathways. Despite this narrow structural span compared to metabolites produced by other marine bacteria, vibrios produce compounds with interesting biological activities. For example the solonamides, cyclic depsipeptides from *P. halotolerans* were found to attenuate virulence in a CA-MRSA strain (**paper 6**).

Many vibrios have multiple lifestyles, including a planktonic (free swimming), sessile (attached to zooplankton or other surfaces), and a pathogenic form.[1] As production of secondary metabolites often confers a selective advantage to the producing organism,[154] the diverse lifestyles of these bacteria are reflected in their metabolic capabilities. There are intraspecies variations in the compounds produced, with different chemotypes potentially reflecting niche adaptation. For example, antagonistic strains of *V. coralliilyticus* isolated from seaweed or sediment during Galathea 3 were found to produce andrimid in high yields, while pathogenic related strains did not have the ability to produce the antibiotic (**paper 5**). The fully sequenced genome of the *V. coralliilyticus* type strain is currently undergoing assembly (Los Alamos National Laboratory).[44] Functional annotation of the genome will reveal whether the andrimid cluster is completely absent from the genome or the genes are merely silenced and to be expressed under appropriate conditions.

The cosmopolitan occurrence of several vibrio metabolites raises the question whether there are unique *Vibrionaceae* metabolites. To date, only nine genomes from vibrios have been fully assembled,[28] mainly pathogenic *V. cholerae* strains.[29] For the future, it will be of outmost interest to extend full-genome sequencing to other vibrios and investigate the prevalence of biosynthetic genes linked to secondary metabolism. Also, this will make it possible to compare homology of biosynthetic genes between diverse producers of cosmopolitan antibiotics. Overall, this might allow insight into the ecological roles of these bacteria and the conditions leading to production of their metabolites.

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5 Overall discussion and conclusions

So far, the marine environment has yielded thousands of bioactive metabolites, including antibacterial, anticancer, and antifouling activities.[1-3] Yet, marine bacteria still represent an emerging source of new natural products and many taxa remain uncharted territory. The challenge of multi-resistant pathogens forces the NP chemists to look to marine bacteria as a source of new chemical diversity for antimicrobial therapy.

The aim of this PhD study was to investigate the production of bioactive secondary metabolites from a global collection of marine *Pseudoalteromonas* and *Vibrionaceae*, with emphasis on the latter as it represents an under-explored source of natural products. Consequently, a primary goal was to provide a robust setup for chemical evaluation of the collected bacteria and to develop methods that facilitate rapid dereplication of the associated extracts. The results obtained have been presented in six papers and elaborated upon in the previous two chapters of this thesis. The broad perspectives and conclusions drawn from this PhD study will be discussed under the following headings.

Marine γ-proteobacteria show potential for production of bioactive secondary metabolites. Members of *Pseudoalteromonas* and *Vibrionaceae* are particular abundant on the surface of various marine macroorganisms, where they form commensal,[4,5] symbiotic,[6] and pathogenic associations.[5,7,8] Several studies have shown that these bacteria are some of the most prolific producers of inhibitory compounds among marine culturable bacteria.[9-11] Despite this high frequency of antagonism, only few species have been investigated for their proclivity to produce secondary metabolites. Especially, the vibrios are an under-explored source of bioactive compounds.

In our global collection of *Pseudoalteromonas* strains, the biosynthetic potential was evaluated through the overall metabolite profiles. We found a clear link between ecological niche and the ability to produce bioactive compounds (**paper 3**). Approximately 88% of the strains that retained antibacterial activity upon re-culturing came from biotic and abiotic surfaces. We found this to coincide with the production of pigments, thus verifying previous observations.[4] In some cases, the pigments themselves had antibacterial activities such as violacein in *P. luteoviolacea* and prodigiosin in *P. rubra*; however, the production of pigments is merely an indication of the general ability to produce

secondary metabolites. The most antibacterial strains were found to belong to the purple-pigmented species, *P. luteoviolacea*, one of the most researched *Pseudoalteromonas* species. Nonetheless, we isolated the potent antibiotic indolmycin for the first time from this species (**paper 1**), underlining the untapped potential of bioactive compounds in *Pseudoalteromonas*. Interestingly, we found certain strains of *P. luteoviolacea* produced an 'antibiotic cocktail' consisting of either violacein/pentabromopseudilin or violacein/indolmycin. We posit that this coordinated expression of different antibiotics will significantly enhance the chance of survival for the producing organism and explain the superior antibacterial activity of this species in our collection.

The biosynthetic potential of *Vibrionaceae* was appraised through *i*) the first complete review of metabolites reported from vibrios (**chapter 4**) and *ii*) metabolite profiling of strains in our global collection showing pronounced antibacterial activity, followed by bio-guided isolation of the active compounds (**paper 4**). We found the antibacterial activity of two *Vibrio coralliilyticus* strains (S2052 and S4053) to be ascribed to the production of the pyrrolidinedione, andrimid. Andrimid has previously been isolated from a *Vibrio* sp.;[12,13] however, this is the first report linking andrimid production to a specific species (**paper 4**). From a single strain of *Photobacterium halotolerans* (S2753), we isolated the antibiotic holomycin (**paper 4**), two novel cyclic tetradepsipeptides called solonamides (**paper 6**), four cyclic tetrapeptides, and five different depsipeptides related to the ngecheumicins[14] (Kjærulff and Månsson, unpublished data, **chapter 4**).[15]

Among compounds reported from vibrios so far, there seems to be a prevalence of non-ribosomal peptides or hybrids thereof, with examples of N-containing compounds produced by NRPS-independent pathways (**chapter 4**). Though covering a limited chemical space, vibrios still produce compounds with attractive biological activities; for example, the cyclic depsipeptide kahalalide F from *V. mediterranei*[16] that is undergoing Phase II clinical trials for the treatment of prostate, lung, and liver cancer.[17] Few vibrios have been fully genome sequenced, but so far none have been functionally annotated with regard to the presence of biosynthetic clusters. Thus, it is still uncertain whether these bacteria represent a novel 'hotspot' of secondary metabolites or whether they are merely scavengers of interesting genes that facilitate their surface-associated lifestyle. Nonetheless, this account has highlighted the pending potential for exploring new bacterial sources of bioactive compounds.

In 1993, one of the pioneers in marine microbial NP chemistry, Professor William Fenical from Scripps Institution of Oceanography wrote in a review on the chemical potential of marine bacteria:[18]

> "*Gram-negative bacteria are not generally prolific in their production of extracellular metabolites. Fermentation yields of less than 1 mg of total organics per liter from dense cultures are common, if not the rule. Anyone wishing to explore this potentially exciting new group of microorganisms should plan for a scaleup to the 100-liter range before anticipating success in this endeavor*".

This statement stems from the very early days of marine microbial NP chemistry and must have been based on mostly unpublished results. Nonetheless, it has turned out to be not far from the truth, as corroborated by results from this PhD study. However, the full extent of Prof. Fenical's statement remains unexplored as literature is biased towards reporting novel chemistry. Only rarely are sources that yield no or only known chemistry described. One outlet is databases like AntiBase that also to some extent include results from conference proceedings. Personally, I think increasing the level of data sharing, 'good' as well as 'bad' results, would be of great value for the microbial natural products community , as it would reduce the risk of rediscovery and futile screening programs.

Production of secondary metabolites in *Pseudoalteromonas* and *Vibrionaceae* may be involved in niche-adaptation. The Galathea 3 collection has provided us with the unique opportunity to study the production of secondary metabolites in marine bacteria on a global scale. A major goal was to investigate the difference between metabolite profiles of 'strain siblings' collected from different geographical locations.

Metabolite profiling of all 101 *Pseudoalteromonas* strain collected during the expedition (**paper 3**) revealed that some species were very homogenous in their metabolite production, regardless of sample location, while others showed both global and local variations. One such species was *P. luteoviolacea*, and interestingly the differences were related to production of antibacterial compounds. Strains S4047, S4054, and S4060 were all collected from the same seaweed sample near Costa Rica (Pacific Ocean), but represented two different chemotypes: violacein/pentabromopseudilin and violacein/-indolmycin producers. Interestingly, strain S2607 collected from a lava rock on the coast of Eastern Australia also showed production of violacein/pentabromo-pseudilin. Investigation of *P. luteoviolacea* strains from other culture collections confirmed that the chemotypes observed were present in strains outside our

Galathea 3 collection, covering geographical locations such as the Mexican Gulf and the Mediterranean. A similar trend was observed in *Vibrionaceae*. *Vibrio coralliilyticus* strains S2052 (sediment, west coast of Australia) and S4051 (seaweed, Costa Rica) had nearly identical metabolite profiles despite their distant sample locations (**paper 4**). Antagonistic activities were ascribed to the production of andrimid; however, andrimid was neither observed in the type strain nor in a closely related *V. coralliilyticus* (**paper 5**). Despite a low degree of chemoconsistency, our studies clearly show that production of secondary metabolites in marine bacteria is more than a strain specific trait.

It has been suggested that secondary metabolites represent functional traits with important ecological roles. Penn et al. (2009) found biosynthetic clusters and other niche-specific genes in *Salinispora* to be concentrated in genomic islands enriched with mobile genetic elements.[19] They suggest that the evolution of secondary metabolites may act as a force driving bacterial diversification.[19,20] Thus, it is reasonable to speculate that *Pseudoalteromonas* and *Vibrio* acquire and retain biosynthetic genes linked to antibiotic production as part of adapting to a specific niche. The number of strains included in our study is merely enough to pose interesting questions considering the link between biogeography and secondary metabolite production. Thus, a study including all 309 *Vibrionaceae* strains collected during the expedition, as well as extending our collection of *Pseudoalteromonas,* is of great interest. Nonetheless, this PhD study shows that investigation of multiple strains of the same species can be a valuable strategy for detection of new compounds and is imperative to uncovering the full biosynthetic potential of a species.

Marine vibrios are capable of producing antibiotics under conditions mimicking their natural environment. Secondary metabolites are hypothesized to play a significant role in the population structure and dynamics of bacterial communities.[21] The exigent questions in chemical ecology focus on how bacteria use chemical substances to interact with eukaryotic hosts and to compete and communicate with other microorganisms. The challenge is to link the chemical information with the natural ecosystem of the bacterium. Studying the physiological conditions, under which interesting molecules are produced, can provide basis for postulating their ecological roles. One approach is to use culture conditions that mimic the natural habitat.

Vibrios have a versatile metabolism and are capable of degrading a variety of complex carbon sources. Thus, we used natural growth substrates, such

as chitin and seaweed extracts, to evaluate the production of antibacterial compounds and other metabolites as part of our screening of *Vibrionaceae*. Both *Vibrio coralliilyticus* (**paper 5**) and *Photobacterium halotolerans* (**paper 6**) were able to produce their antibacterial compound, andrimid and holomycin respectively, when using chitin as the sole carbon source. Also, *V. coralliilyticus* was able to produce andrimid on extracts of two species of seaweed, *Fucus* and *Laminaria* (**paper 5**). As vibrios are often abundant in chitinous microenvironments such as on the surface of zooplankton and crustaceans, our studies suggests that these compounds can be produced in the natural environment. However, we do not know at which concentrations or how other environmental factors influence their production.

The overall metabolic response to the growth conditions was different for the two bacteria. When grown on chitin, *V. coralliilyticus* produced only andrimid and turned down production of other metabolites (**paper 5**). Interestingly, the yield of andrimid per cell was increased two-fold compared to the standard laboratory medium. Conversely, *P. halotolerans* sustained production of all metabolites, and potential novel compounds were observed only on the chitin medium (**section 3.2.2**). This different phenotypic response can indicate different niche adaptations or different ecological roles of the compounds produced. Chitin has been reported to have both stimulating and inhibitory effects on antibiotic production in *Streptomyces*[22] and *Pseudoalteromonas,*[23] corroborating that natural substrates have multiple effects. Without the appropriate ecological context, circumspection is required in drawing ecological interferences. Nonetheless, our studies show that chitin and other natural growth substrates can be a promising strategy for activating biosynthetic pathways in marine bacteria.

Marine bacteria demonstrate potential for production of specific QS antagonists. The selective force that is inherently linked to growth inhibition eventually engenders antibiotic resistance.[24] Turning off virulence gene expression exogenously, and thereby quenching QS regulated virulence, may represent an alternative strategy for the treatment or prevention of bacterial infections.[25-27] To further explore this avenue in antimicrobial therapy, we have included assays based on QS systems in both Gram-positive and Gram-negative bacteria in our Galathea 3 screening setup.

The QSI selector assays used for identification of inhibitors of QS in Gram-negative bacteria is based on the two homoserine lactone-mediated systems, *luxR* from *Vibrio fischeri* (QSIS1) and *las/rhl* in *Pseudomonas aeruginosa*

(QSIS2).[28] The latter controls the expression of various virulence factors such as proteases and rhamnolipids.[29] We found indolmycin-containing E-SPE fractions from *Pseudoalteromonas luteoviolacea* to have good activity in the QSIS1 assay (Månsson & Holm Jacobsen, unpublished data, **section 3.3.2**), and the activity was verified by testing pure, isolate compound as well as a commercial standard. The effect on LasB and RhlA (QSIS2) was tested in a dose-dependent manner, which showed repression of *lasB* at low concentrations. At increased concentrations, indolmycin was strongly growth inhibitory of *Pseudomonas aeruginosa* and thus not a QS inhibitor in *sensu stricto*. These results are in concordance with other studies that found antibiotics at sub-inhibitory concentrations to affect QS-regulated gene expression.[30-32]

The screen for inhibitors of Gram-positive QS is based on the *agr* system in *Staphylococcus aureus.*[33] The assay monitors three key virulence genes, *spa, hla,* and *rnaIII* under the control of *agr*.[34] These genes encode virulence factors such as extracellular toxins and cell surface adhesion factors.[35] From a strain related to *Photobacterium halotolerans* S2753, we isolated two new cyclic depsipeptides, designated solonamide A and B, as potent inhibitors of this system (**paper 6**). Interestingly, the decreased expression of virulence genes was pronounced in the highly virulent USA300 strain, which is a predominant community-acquired MRSA strain in the United States.[36] High Agr activity is suspected to be a main contributor to the aggressiveness of the strain.[37,38] Thus our findings suggest that quorum sensing inhibition could be an option for treatment of *S. aureus* USA300 infections. The similarity in structure and size of the solonamides and the cognate autoinducing peptides (**paper 6**) imply that they interfere with transmission of the QS signal by competing for binding to the transcriptional regulator proteins. However, it will be necessary to establish their exact mechanism of action. Also, the ability of the solonamides to control *S. aureus* infections in appropriate animal models is of considerable interest.

Many genes are under QS control in both Gram-positive and Gram-negative bacteria, and thus it is not a simple drug target.[39,40] For example, biofilm formation in *S. aureus* has been linked to low QS activity,[41] and so there is a risk that the use of QS inhibitors could lead to decreased susceptibility of traditional antibacterial drugs. Also, it is still unknown how QS inhibition will affect the overall fitness of a pathogen under *in vivo* conditions and thus pose a selective pressure for the development of resistance.[42] Studies have indicated that bacteria can circumvent a QS blockade by over-expressing receptor genes or by changing their specificity.[39] However, as virulence interference is usually species-specific, the overall risk is considered to be significantly lower.[26]

Cosmopolitan occurrence of microbial antibiotics increases the risk of rediscovery. Many secondary metabolites appear to have crossed boundaries imposed by both geography and ecology.[43] Thus, it is not uncommon to isolate compounds previously described from non-marine microorganisms from marine bacteria.[44] Production of secondary metabolites can be seen as a functionally adaptive trait.[45,46] Hence, phylogenetically unrelated species may have arrived at the same antibiotics independently because of the selective advantage that these bioactive compounds confer.[43,47] Some antibiotics can be found widely distributed among distant species and even taxa, an extreme example being the cephalosporins that can be found in actinomycetes, γ-proteobacteria, and fungi.[48]

In this PhD study, the known antibiotics indolmycin (**paper 1**) and holomycin (**paper 4**) have been isolated for the first time in Gram-negative bacteria. Both compounds have originally been isolated from terrestrial actinomycetes. They add to the list of violacein, pentabromopseudilin, prodigiosin (**paper 3**), and andrimid (**paper 4**), as examples of cosmopolitan antibiotics found in *Pseudoalteromonas* and *Vibrionaceae*. Horizontal gene transfer represents a likely mechanism for acquiring such biosynthetic gene clusters. Comparison of the andrimid gene cluster in *Vibrio* and *Pantoea* showed that the biosynthetic domains are perfectly interleaved, consistent with exchange of the whole PKS-NRPS cluster.[49] Also, the cluster is flanked by a transposase pseudogene suggesting that it has been introduced by horizontal gene transfer.[49] This genomic promiscuity is not restricted to members of *Pseudoalteromonas* and *Vibrionaceae* in the marine environment. Five of eight isolated compounds from the actinobacterial genus *Salinispora* have been isolated from unrelated taxa.[19] This underlines that bioprospecting *per se* is not a successful strategy for discovering novel chemistry.

Efficient dereplication is paramount in the search for new microbial natural products. Bacterial NP extracts are often characterized by high structural complexity and diversity, the presence of multiple bioactive compounds, and many metabolites produced in very low yields. To make matters more complicated, high plasticity of bacterial genomes dissolves any taxonomic boundaries for secondary metabolite production. Hence, to unravel a bacterium's full potential for producing novel compounds can be a daunting task.

Statistical analysis based on data from our in-house collection of 718 secondary metabolites from filamentous fungi showed that LC-MS is a robust method for front line dereplication of microbial extracts (**paper 2**). High

confidence mass spectral data can be obtained with a combination of positive and negative ESI-MS, using different diagnostic ions to validate the assignment of the molecular ion. For 93% of the compounds in the in-house database, LC-MS could unambiguously assign the molecular ion. In order to correctly translate the mass of this ion into a single molecular formula, we found that the mass accuracy needs to be below 1 ppm based on structures reported in AntiBase 2008. But even with the correct molecular formula, a search in AntiBase or other commercial databases will seldom produce a definitive answer. A characteristic UV spectrum can help narrow down the number of candidates, but for peptides containing mainly non-aromatic amino acids and other non-chromophoric compounds this information is futile. For peptides, which constitute a common compound class in bacteria (**chapter 4**),[50,51] a primary sequence can be obtained from MS/MS which greatly facilitates the dereplication.

We realized that, in most cases, a second level of dereplication is imperative for evaluating bacteria due to the complexity of the extracts. A large part of this PhD study was concerned with the development and validation of a so-called explorative solid-phase extraction (E-SPE) protocol for dereplication of complex extracts (**paper 1**). The method is based on a set of four SPE columns with orthogonal selectivities that can provide valuable information about the size, charge, and polarity of the active components of an extract. We found especially the presence of ionizable functionalities to be highly discriminatory for the dereplication of microbial compounds. Though not as discriminatory as NMR-based dereplication methods, we found E-SPE to be more rewarding for highly complex, low-output extracts such as those of marine bacteria.

E-SPE successfully integrates the process of extract prioritization, dereplication, mapping of biological activities, and formulation of a purification strategy based on small amounts of crude extract. Since the steps of E-SPE are completely orthogonal, it allows access to potential new compounds and activities. We found that our hit rate in the QSI assays was markedly increased when testing E-SPE fractions rather than crude extracts. This is probably due to the more subtle QSI effects being camouflaged by antibacterial activity against the target organisms. Additional dividend to the E-SPE approach is the potential to design and test the optimum purification strategy before engaging in large-scale experiments, thus highlighting potential pitfalls and greatly improving the yield of purification. By having a more rational approach to the chemical screening process, we have saved a lot of time and effort. Moreover, the E-SPE approach presents a consistent setup that is transferable between different organisms.

Dereplication needs to be performed at various stages of the discovery process, and should not be seen as a single process but rather a continuous critical evaluation for prioritization of both extracts and compounds. This is supported by a triad of analytical techniques each with their obvious advantages and limitations. The success of any dereplication will depend on choosing the right technique based on the difficulty and nature of the problem. The dereplication methods used vary with the facilities available, the type of organism, and the overall screening setup. Many favorable dereplication methods are available; however, a prerequisite for implementing a technique as part of a screening setup is that the equipment is available for routine analysis. Therefore, a dereplication method will mostly represent a compromise based on the analytical facilities of a given lab. If the screening setup is based on traditional growth inhibition assays, known candidates can often be excluded based on a tentative identification alone. However, when exploring new targets for antimicrobial therapy, for example, both novel and known compounds need to be purified and structure validated in order to verify the observed bioactivity if no commercial standard is available. Still, dereplication proactively decreases the number of fractions in a natural product extract that need to enter the purification process, which saves valuable time and resources.

Great database resources are currently available for the dereplication of natural products; however, with the steady increase in isolated compounds, the level of data sharing in the NP community needs to be augmented. Pivotal information, such as ^{13}C and ^{1}H NMR chemical shift values, UV spectra, and MS/MS fragmentation patterns, is currently unavailable or incomplete in commercial databases. Compiling this data will enable rapid recognition of not only known compounds but also their analogues. Like the microbiologist share strains and gene sequences related to published data, the chemist should share the chemical data related to reported structures, which should be stored in central places such as Beilstein (Elsevier), Chemspider (Royal Society of Chemistry), or Chemical Abstracts (American Chemical Society). Though this data collection may be cumbersome, its benefits to the natural products community speak for themselves and should motivate a joint effort to complete the upgrade.

Perspectives and concluding remarks. To preserve microbial natural products as a viable option for drug discovery, the use of a multidimensional screening strategy is imperative:

- Many marine bacterial taxa remain under- or unexplored for their proclivity to produce bioactive compounds. While screening of phylogenetically novel sources does not bring a guarantee for novel chemistry,[43] bioprospecting is still a simple strategy to enhance diversity in a screening program.[52] Functional metagenomic libraries provide a means to probe the uncultured majority of marine microbial life, and constitute a promising tool for drug discovery.[53,54]

- Culture conditions (including co-culture experiments), the use of elicitors, and the OSMAC approach will help accessing compounds that are not constitutively expressed.[52]

- Traditional growth inhibition assays measure antibacterial activity with no distinction regarding the exact target or mode-of-action[55] and will inevitably lead to a high rate of rediscovery due to the cosmopolitan occurrence of many antibiotics.[43] Extending a screening program with target-based whole-cell assays that interrogate one target gene or a group of targets at a time,[56,57] such as virulence genes,[58] forms a rational approach to future drug discovery.

- Testing a mixture of crude extracts, pre-fractionated samples, and pure compounds is necessary to obtain a realistic hit-rate, with a minimum of false positives and false negatives.[59-61]

- Maintaining an array of analytical techniques for rapid dereplication of known compounds and their analogues is imperative to reducing time from hit to lead. Continuous reduction of the scale on which it is possible to do structural elucidation makes it possible to access previously overlooked candidates.[62]

- Exponentially growing sequence information allows the discovery of cryptic gene clusters. *In silico* structure predictions combined with synthetic chemistry,[63] heterologous expression, and combinatorial biosynthesis[64,65] are just some of the tools for accessing and exploiting the biosynthetic potential of marine bacteria through genomic data.

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Paper 1

"Explorative Solid-Phase Extraction (E-SPE) for Accelerated Microbial Natural Product Discovery, Dereplication, and Purification"

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Paper 1

Explorative Solid-Phase Extraction (E-SPE) for Accelerated Microbial Natural Product Discovery, Dereplication, and Purification

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Microbial natural products (NP) cover a high chemical diversity, and in consequence extracts from microorganisms are often complex to analyze and purify. A distribution analysis of calculated pK_a values from the 34390 records in Antibase2008 revealed that within pH 2−11, 44% of all included compounds had an acidic functionality, 17% a basic functionality, and 9% both. This showed a great potential for using ion-exchange chromatography as an integral part of the separation procedure, orthogonal to the classic reversed-phase strategy. Thus, we investigated the use of an "explorative solid-phase extraction" (E-SPE) protocol using SAX, Oasis MAX, SCX, and LH-20 columns for targeted exploitation of chemical functionalities. E-SPE provides a minimum of fractions (15) for chemical and biological analyses and implicates development into a preparative scale methodology. Overall, this allows fast extract prioritization, easier dereplication, mapping of biological activities, and formulation of a purification strategy.

For the purification of natural products, specific chemical information on the target compounds prior to purification is vital for the success of the purification.[1,2] If no prior knowledge about the target compounds is available, a purification strategy is normally developed through trial and error or by standard fractionation procedures and often guided by one or more bioassays. Many natural product (NP) laboratories have developed internal strategies for standardized extract screening and purification,[3−5] while in other cases, only parts of the overall setup have been published as the techniques have probably evolved over many years and the rationale behind them has not been disclosed.[6]

For the most part, modern NP purification methods are based on reverse-phase (RP)[4,7] separations due to high capacity, recovery, reproducibility, and chromatographic resolution compared to most separation methods.[2] However, when faced with complex extracts containing many components, a purification strategy based solely on RP can lead to problems. This may involve the purity of the end products, the overall recovery from the extract, or at worst, permanent loss of activity due to instability or degradation. This is especially a problem with low yielding extracts typical of those arising from marine microorganisms.[8] For this type of extract, orthogonal purification strategies are required; classically, this can be achieved by combining RP with normal-phase chromatography on silica gel (low cost)[2] or alternatively using bonded phases such as polyethyleneimine[9,10] or diol.[11,12] Ion-exchange has been used less frequently[13−16] even though many natural products contain ionizable groups.

In contrast to preparative chemistry, matrix-dependent orthogonal solid phase extraction (SPE) purification strategies are widely used in trace analysis to complement the almost universal analytical RP-LC methods used.[17] This is essential to effectively remove coeluting interferences as well as the major constituents of the matrix. Mixed-mode sorbents,[18,19] multifunctional columns,[20] and especially ion-exchange SPE[21,22] are widely applied to ensure high and consistent recoveries.

Targeted exploitation of chemical functionalities in the work with novel compounds has not been used frequently prior to preparative isolations. Only two papers have described methods for the preliminary chemical characterization of NP extracts to be used as part of the development of purification strategies. Samuelsson et al. used Sephadex G25, anion- and cation-exchangers, and a set of solvents for liquid−liquid partitioning to investigate the size, charge, and polarity of the active constituents in aqueous plant extracts,[13] while Cardellina et al. used a combination of Sephadex G25 and RP resins.[23] Both groups used their methods to evaluate bioactive, aqueous extracts of high complexity. In addition, by using a bioactivity elution matrix Cardellina et al. were also able to use the method for dereplication and prioritization of extracts.[23]

In microbial NP extracts, multiple biosynthetic pathways are often represented,[21,24−28] resulting in complex extracts. To deal with this, a standard protocol capable of exploiting the different chemical functionalities is required. Thus, we have developed "explorative solid-phase extraction" (E-SPE) where a set of SPE columns with orthogonal selectivities are used to rapidly explore the optimum purification strategy on a small scale in the exploratory stage of the discovery process, which has the potential to be transferred to a preparative scale. Also, by supplying information on the presence of ionizable functional groups, the analytical (e.g., LC-MS) dereplication of candidates is facilitated. Here, we present the use of E-SPE as a standard procedure that is compatible with a broad series of bioassays and enables the use of a standardized screening method transferable between different organisms and bioactive NP targets. Overall, this allows a rational approach to the purification process that is independent of the experience and intuition of the chemist and integrates the process of extract prioritization, dereplication leading to the mapping of biological activities, and formulation of a purification strategy.

Results and Discussion

To determine the incidence of microbial natural products with charged functionalities, a batch calculation using the Advanced Chemistry Development pK_a suite was conducted on all records in AntiBase 2008,[29] resulting in 34390 valid records. This revealed (Figure 1) that 52% of all compounds reported had an ionizable functionality (within pH range 2−11, see Figure 2). This confirmed the potential for incorporating ion-exchange chromatography as an integral part of the separation procedure. Three different ion-exchangers were included in the overall setup. An approach based on the cation exchange of amines was perceived to be the most discriminatory prospect followed by anion exchangers for carboxylic

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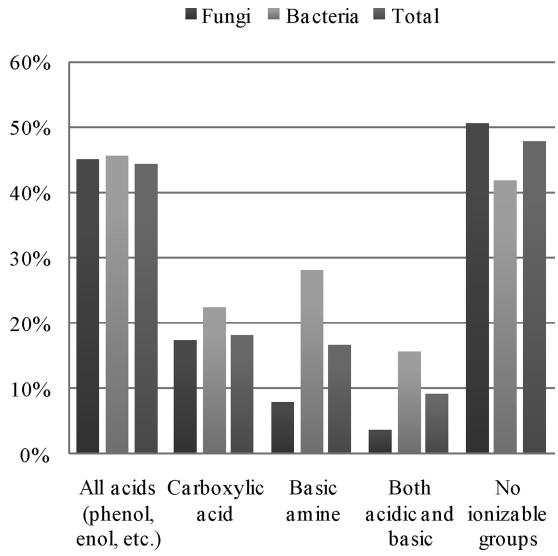


Figure 1. Distribution of microbial natural products in AntiBase 2008 with charged functionalities within pH range 2−11, permanently charged groups such as sulphates, phosphates, and tertiary amines as well as potential tautomeric forms excluded. Statistics are based on theoretical pK_a values calculated using Advanced Chemistry Development pK_a suite.

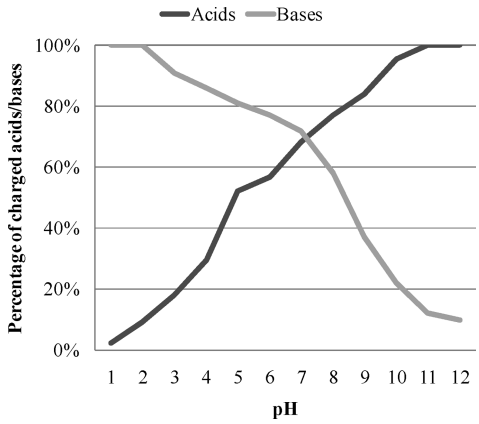


Figure 2. pK_a Cumulative distribution of the ionic form of acids and bases in Antibase 2008 at a given pH value, based on their calculated pK_a values. Theoretical pK_a values calculated using Advanced Chemistry Development pK_a suite. The statistics includes permanently charged groups such as sulphates, phosphates, and tertiary amines but excludes potential tautomeric forms.

acids. Thus, strong anion- and cation-exchangers (SAX and SCX) were included to reveal the presence of carboxylic acids and amines. A mixed-mode polymeric RP anion-exchanger (Oasis MAX) with a poly(divinylbenzene-co-vinylpyrrolidone) backbone was included as it has been shown not only to retain acidic compounds with a carboxylate functionality but also weaker anions of phenols and tautomeric enols; due to the polar polymeric backbone, it can retain these close to the ionic groups.[18,19] On the basis of observations from analysis of fungal metabolites in food and feed samples, it was speculated that enols, phenols, and other compounds with acidic functionalities could be differentiated from carboxylic acids by comparing SAX and MAX elution patterns. In addition, the MAX column could also provide information about the relative polarity of the compounds in the extract, thereby keeping down the number of columns and fractions as an RP column was now not needed. A Sephadex LH-20 column (polymeric cross-linked dextran gel for size-exclusion) was included to give information about the relative size of the compounds. These four orthogonal columns provided a rational compromise to obtain the necessary information on size,

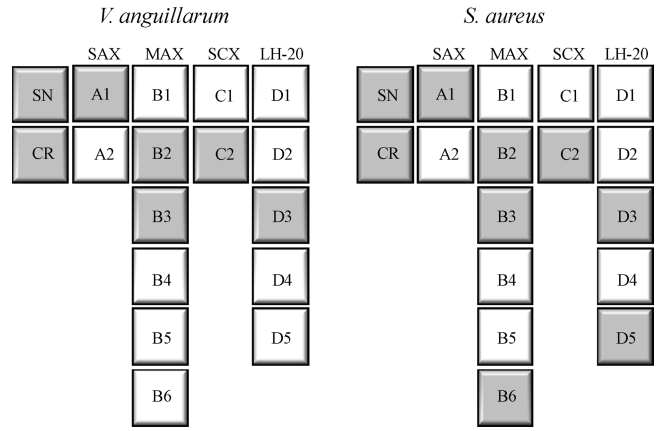


Figure 3. E-SPE profiles of the ethanolic extract of *P. luteoviolacea* obtained in well-diffusion assays with *V. anguillarum* and *S. aureus.* The matrix represents fractions from the four SPE columns as well as their corresponding supernatant (SN) and crude extract (CR). Gray = active; white = no activity observed. SAX fractions: A1 = unretained bases and neutrals, A2 = retained acids. MAX fractions: B1/B2/B3 = polar/medium polar/apolar unretained bases and neutrals, B4/B5/B6 = polar/medium polar/apolar acidics. SCX fractions: C1 = unretained acids and neutrals, C2 = retained bases. LH-20 fractions: D1−D5 = fractions of decreasing molecular size (band-based). Violacein identified in fractions A1, B6, C2, and D5. Potential new bioactive identified in fractions A1, B2, B3, C2, and D3.

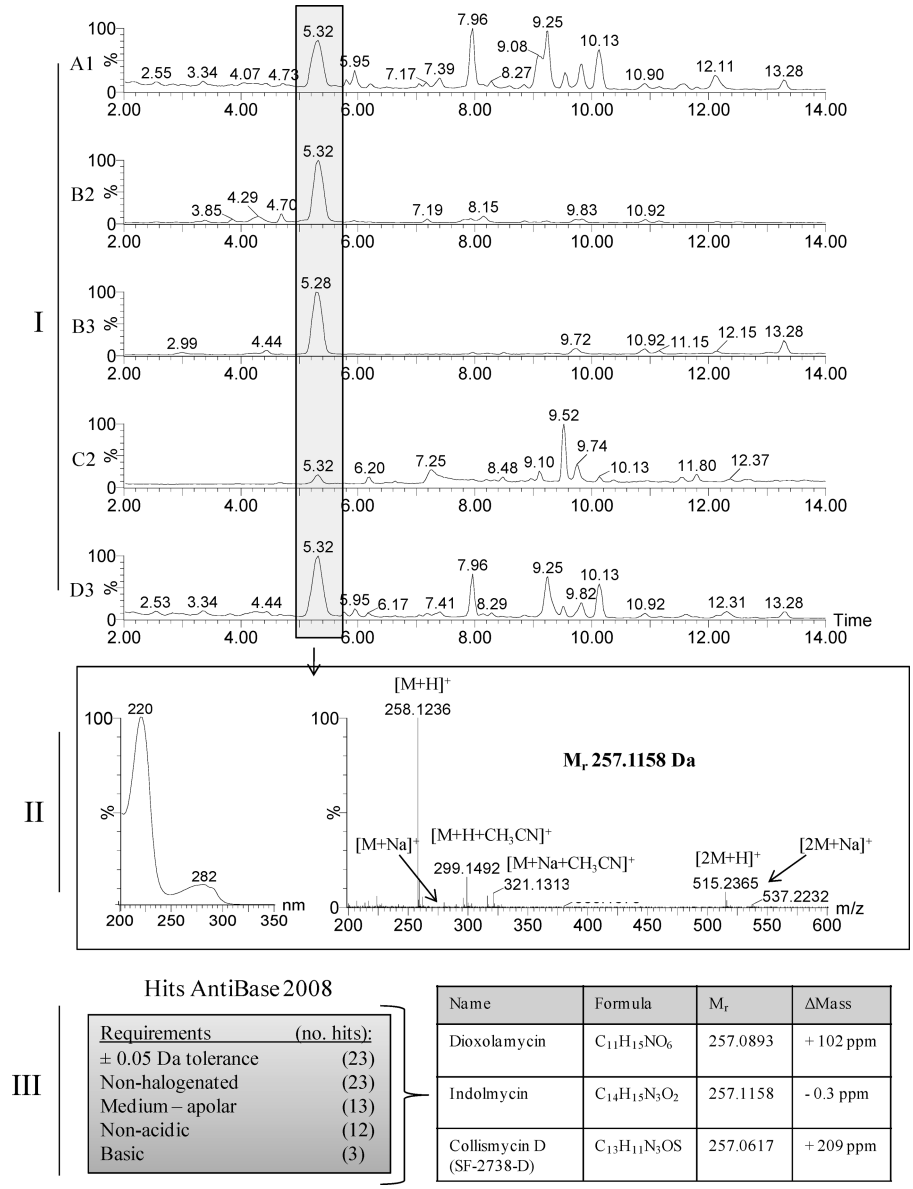
charge, and polarity of the extract components while still generating the minimal number of fractions.

Beause the goal was to develop E-SPE as a standard operating procedure when dealing with new microbial extracts, the procedure was tested on both fungal and bacterial extracts, including extracts of marine bacterial origin. Plant extracts were not included, as an existing range of standardized prefractionation or partitioning steps are established and targeted to separate the major compound classes present in plants such as chlorophylls, polyphenolics, tannins, and saponins.[30−32]

Typically 0.5−2 mg dry extract aliquots were applied to each column. The specific extraction procedure varied with the type of organism and matrix. For example, media with high salt content should be freeze-dried and redissolved in EtOH prior to ion-exchange,[33] as the ions from the media will impair the binding (salting-out) of possible target compounds on the SAX and SCX columns.

A total of 15 fractions were generated from each extract and submitted for biological testing with part of the crude extract/culture supernatant and recombined fractions from each of the columns to reveal potential instability issues or synergy effects. To track false positives, a blank medium sample was subjected to the same extraction and fractionation procedures as the cultures.

The assay results were organized in a bioactivity matrix similar to that used by Cardellina et al.[23] (Figure 3). Visual interpretation of this matrix made it possible to map the active components by comparing the results from different assays. Active and nonactive fractions were subjected to comparative dereplication by LC-HRMS (Figure 4) by cross-referencing MS and UV peaks between fractions. Peaks only appearing in active fractions and not in nonactive fractions are potential candidates for the observed bioactivity. Afterward, the corresponding MS and UV spectra were extracted, adduct patterns established, and the accurate mass data determined using reported analyses.[34] The accurate mass was used as query in a database search (AntiBase, AntiMarin, or similar). The resulting candidates were assessed based on their: (i) mass accuracy and isotope pattern, (ii) match between the acquired UV spectrum and the reported UV data, (iii) observed retention time compared to structure, LogD, molecular size etc., (iv) taxonomic

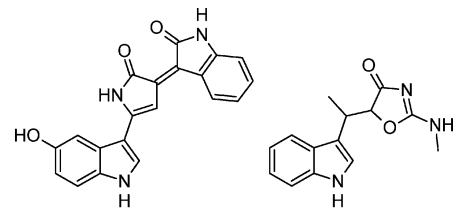


Name	Formula	M_r	ΔMass
Dioxolamycin	$C_{11}H_{15}NO_6$	257.0893	+ 102 ppm
Indolmycin	$C_{14}H_{15}N_3O_2$	257.1158	- 0.3 ppm
Collismycin D (SF-2738-D)	$C_{13}H_{11}N_3OS$	257.0617	+ 209 ppm

Figure 4. Comparative dereplication of E-SPE fractions from *P. luteoviolacea* active against *V. anguillarum*. (I) LC-MS total ion chromatograms (ESI^+) of active fractions A1, B2, B3, C2, and D3. (II) Extracted UV and ESI^+ MS spectra from common peak with retention time RT = 5.32 min, providing an accurate mass (monoisotopic) M_m of 257.1158 Da. (III) Candidates from AntiBase 2008 that satisfy all functional criteria elucidated by E-SPE. Total number of hits noted in parentheses.

data, (v) match between charged functionalities and ion-exchange properties, and (vi) ESI^- and ESI^+ adduct patterns and the strongest ionization mode. The final list of possible targets indicated the likelihood of the candidates being novel structures. When the potential targets were identified, it was possible to develop a suitable procedure for purification, preferably based on selective ion-exchange properties. As final validation before scaling up, the chosen optimized combination of columns was tested in series while following the concentration of bioactivity.

E-SPE has been introduced as a standard screening procedure in our lab for the evaluation of new extracts. Many extracts have been screened, and herein we present two cases, one bacterial and one fungal, which represent some of the experience we have gained through using this protocol. To validate our method, we applied E-SPE with an optimized fractionation protocol to an extract of the marine bacterium *Pseudoalteromonas luteoviolacea* grown in a rich medium containing 3% sea salts. *P. luteoviolacea* is a known producer of several antibacterial compounds.[35−41] Under the conditions used, it generated a highly complex extract which was further complicated by high levels of leftover media components. This made full dereplication on the data from the crude extract a difficult task. The ethanolic extract was dominated by the purple pigment, violacein, which has a broad range of antibacterial activities, especially on Gram positive bacteria.[36,42,43] E-SPE bioactivity profiles (Figure 3) were obtained for inhibitory activity against the Gram-negative bacterium *Vibrio anguillarum* and the Gram-positive bacterium *Staphyloccocus aureus.*



Violacein Indolmycin

Comparison of the three profiles showed the presence of at least two bioactive compounds in the extract. As expected, violacein was

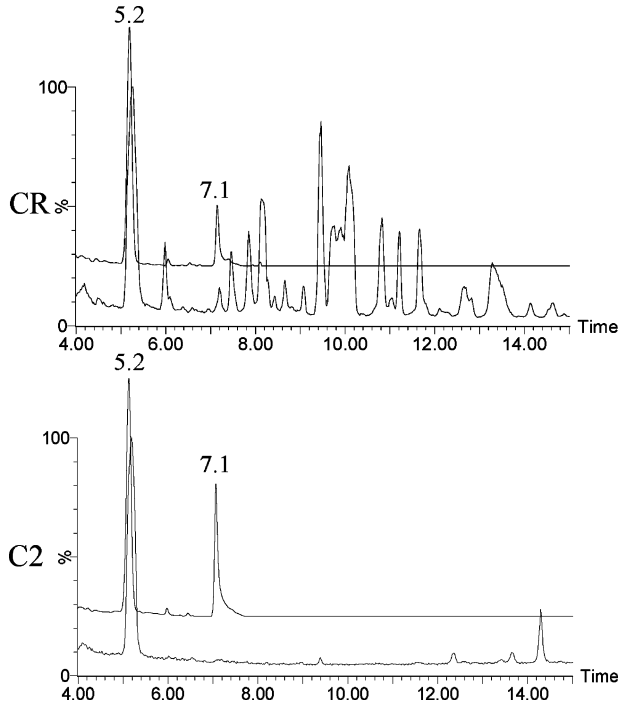


Figure 5. LC-DAD-MS chromatograms with UV (200−700 nm) above total ion chromatogram (*m/z* 100−900 Da) of *P. luteoviolacea* crude (CR) extract (top) and retained (C2) SCX fraction (bottom). Indolmycin (RT 5.2) and violacein (RT 7.1) are selectively retained on a strong cation-exchanger.

identified by LC-HRMS in fractions A1, B6, C2, and D5 that were active against *S. aureus*. This distribution was consistent with the functional groups present in the structure.

A second bioactive compound was observed in both assays, with a recognizable activity pattern of fractions A1, B2, B3, C2, and D3. On the basis of its distribution in E-SPE fractions, the compound was expected to be nonacidic, medium to apolar, medium sized with a basic functionality. By comparative dereplication (Figure 4), an accurate mass of 257.1157 Da was elucidated. No previously reported compounds from the genus *Pseudoalteromonas* or related genera were found to possess these properties. However, indolmycin,[44,45] a known antibacterial from *Streptomyces*, was found to be a likely candidate, as determined by a search in AntiBase. Indolmycin has not previously been reported to be produced by Gram negative bacteria. Therefore, for absolute identification of the compound, a large-scale extract was made and subjected to cation-exchange (Figure 5) followed by size-exclusion as indicated by the E-SPE profile (Figure 3). This led to the isolation of both violacein and indolmycin. Both structures were verified by NMR spectroscopy and matched with previously published data.[46−48] The finding of these two compounds was consistent with the original E-SPE bioactivity matrix.

As part of the method validation, the E-SPE protocol was also tested on a *Penicillium roqueforti* extract. Like the *Pseudoalteromonas luteoviolacea*, *P. roqueforti* yielded a complex extract with a diverse range of metabolites.[49,50] This species has been thoroughly investigated for metabolite production because it is a very common contaminant of food and feed as well as a starter culture for blue cheese. As *P. roqueforti* is prolific in production of organic acids, phenols, and enols, the two anion-exchangers were suitable for the early fractionation of extracts from this fungus.[51] The mixed-mode anion exchanger (MAX) selectively retained all acids, including acidic enols like the andrastins (pK_a 4.5). Comparing the two fractions eluted with 60% organic, unretained bases/neutrals (B2) and acids (B5), respectively, it was clear that the MAX column as a first step of purification markedly simplified the extract (Figure 6). The peaks across the chromatogram were essentially bisected between B2 and B5 with no overlaps between the compounds in the nonacidic and acidic fractions (Figure 6). The MAX separation revealed a series of potential new compounds masked under the peaks of the major metabolites, roquefortine C and mycophenolic acid, which dominate the chromatogram of the crude fraction before partitioning. These new compounds could then be purified using an RP strategy with much improved recoveries, potentially after an SCX step to remove alkaloids like the roquefortines. This underlined the usefulness of employing an orthogonal purification strategy.

The E-SPE approach offers several advantages when dealing with complex extracts. First of all, E-SPE enables the formulation of a purification strategy based on small amounts of crude extract. Then, by using a four-column strategy, it is possible to design preparative purification steps that selectively retain the target candidate (and related compounds) or remove unwanted components. Furthermore, when working with bioactive extracts, it is possible to test the first steps and track the concentration of bioactivity by putting the columns in series. This procedure can be directly translated into a larger scale purification process with high reproducibility as was demonstrated with *P. luteoviolacea*. These aspects of the E-SPE approach are considered in more detail under the following headings.

Dereplication. Successful dereplication of natural product extracts is imperative in the discovery process but can be time-consuming because it requires assessment of the candidates and retrieval of the papers describing all candidates.[24,52] E-SPE accelerates dereplication by reducing the overall number of peaks to be identified and many of the potential candidates in a database search can be eliminated directly based on their ion-exchange properties. E-SPE and *comparative* dereplication makes it, in simple cases, possible to carry out target-guided isolation rather than bioguided fractionation, thereby reducing the need for bioassay support during the isolation process.[53] Further advantages can be gained by using an automatic comparison of chromatograms, e.g., multivariate tools based on principal component analysis (PCA) within the software packages of the major MS vendors. By using E-SPE, it is possible to exploit the sensitivity of biological assays to extract information about the active components and the chemical functionalities. This is of particular importance when working with natural products that are poorly ionized in all MS modes and/or for minor components present below the detection threshold.

Mapping Biological Activities. E-SPE serves as a valuable prefractionation step before biological testing. Bugni et al.[4,54] and Appleton et al.[55] demonstrated that prefractionation can extensively reveal masked candidates in a bioassay (with up to 80% of the candidates being masked in the original extract) and reduce false positives.

Because the steps of E-SPE are completely orthogonal, it allows access to potential new compounds and activities, for example, in cases where: (i) several compounds are responsible for the observed bioactivity, (ii) one compound is responsible for multiple bioactivities, i.e. privileged structures,[56,57] or (iii) if several compounds with different activities are present in the extract.

The potential differentiation was exemplified in the case of *P. luteoviolacea* (Figure 3); violacein was responsible for the antistaphyloccoccal effect observed, whereas indolmycin displayed multiple types of bioactivity (antistaphyloccoccal and antivibrio). The presence and bioactivity of indolmycin could easily have been overlooked in the absence of any kind of prefractionation.

Scaleup and Optimization. By using E-SPE as the first step in optimizing a small scale purification strategy, it is possible to investigate the success or failure of individual purification steps prior to working with a much larger extract. Because all the columns are readily accessible for scaleup, it is possible to directly transfer the strategy to preparative scale, as demonstrated for *P. luteoviolacea*. Sephadex LH-20 is probably the only column that cannot

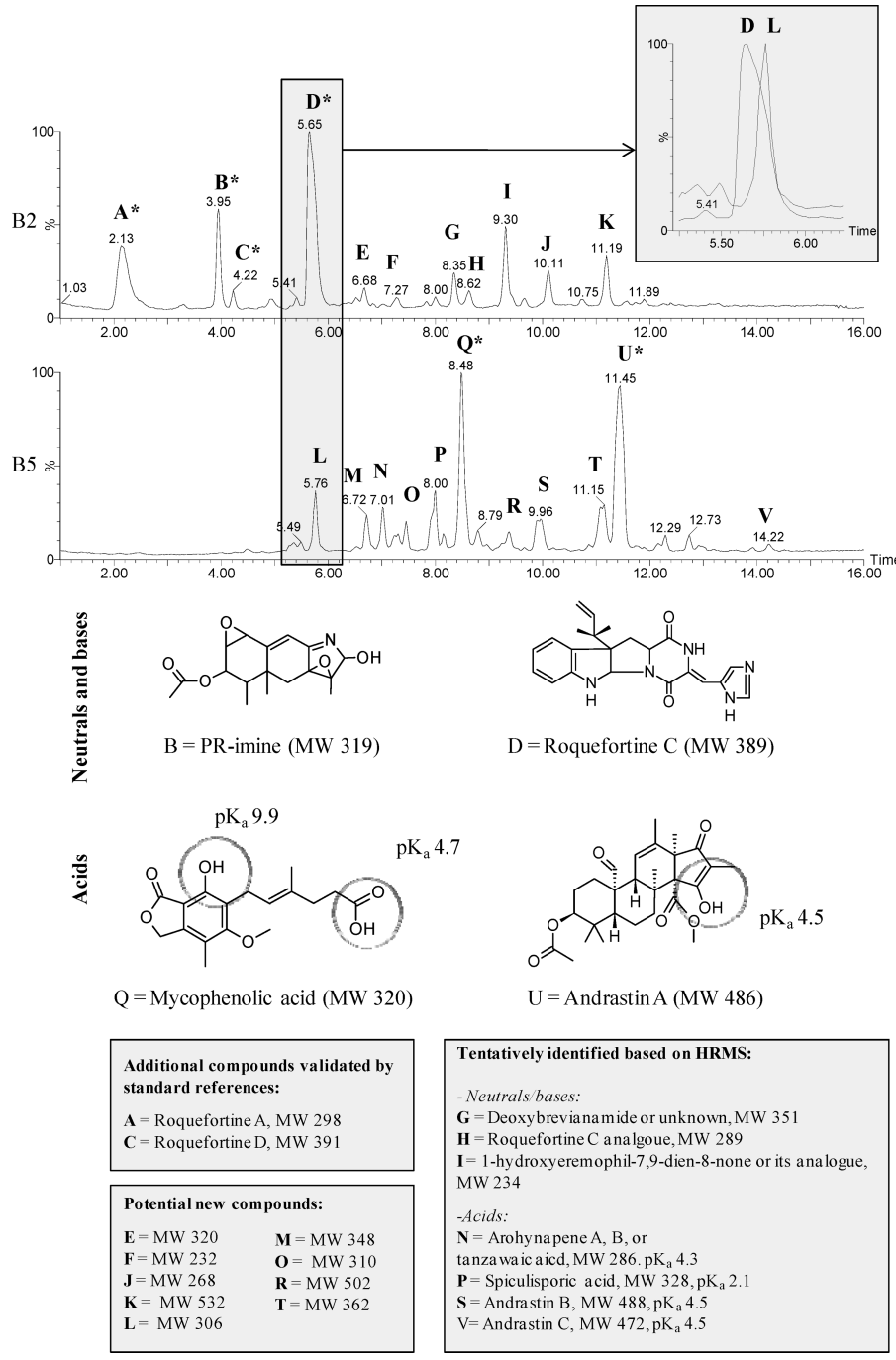


Figure 6. LC-MS total ion chromatogram (*m*/*z* 100−900) of *P. roqueforti* E-SPE fractions from MAX column. B2 = unretained neutrals and bases eluting with 60% MeOH; B5 = retained acids eluting with 60% MeOH and formic acid. Compounds with "*" are identified through comparison with available reference standards. The rest of the compounds were tentatively identified from AntiBase 2008 through E-SPE properties, UV spectrum, and accurate mass.

be directly translated into a large-scale method as the separation is dependent on the column dimensions, linear flow rate, loading of the sample, etc.[58] However, E-SPE still indicates whether this solvent and time-consuming chromatographic step would be worthwhile.

It is important to note that this protocol is only the first step to determine the strategy being pursued. A second optimization experiment could be necessary when other columns or slight variations in the loading or elution of the compounds are to be evaluated. For example, if highly polar compounds are encountered, redissolving the extract in H_2O then loading on Strata-X or Oasis HLB columns may be a way to retain the bioactives. Compounds not retained under these conditions would need to be targeted by HILIC, Sephadex G-10, or other hydrophilic separation approaches.[59]

Optimization of the pH for binding acids and bases may be another important parameter to consider before applying the E-SPE strategy to a large-scale extract. This is of special importance when dealing with a pH labile target, or extracts containing a large fraction of charged compounds. Compounds such as (open lactone form) statins and homoserine lactones are selectively retained on Oasis MAX columns (80−100% recovery) at a 0.5 pH unit below the pK_a even though only 32% (= 10^{pK_a-pH}) theoretically should be charged. Thus, the selectivity and recovery of an ion-exchange step can be further improved by exploiting this dynamic equilibrium during ion-exchange.

Cost and Access. E-SPE saves time and resources when employed on complex extracts. The method represents an inex-

pensive and simple type of chemical screening that can also be used in laboratories with limited access to chemical equipment. The cost per extract is approximately 6−8 USD plus costs for analytical LC-DAD, LC-MS, LC-NMR, etc. When running SAX, MAX, and SCX cartridges, it is easy to run up to 14 extracts in parallel and these columns are readily accessible for automation in microtiter format, as whole plates, or plates with different sorbents that are commercially available. A lower number is recommended for Sephadex LH-20 due to a higher variability in flow rates through the small self-packed columns.

Even though E-SPE is fast, reproducible, and easy to use, there are a few issues to be aware of: There should be good correlation between the sensitivity of the assay used and the extract amounts to avoid false negatives, but more importantly, to track false positives, a blank medium sample should be run in parallel with the other E-SPE samples as one will also concentrate interfering media components.

Some knowledge of the sample matrix is necessary as high levels of salts and fats give rise to problems in the sample run and load, and potentially cause interference in the bioassays. This might require adjustments to the sample pretreatment by: (i) freeze-drying extracts and redissolving in EtOH, (ii) liquid−liquid partitioning, (iii) Sephadex G-10 size chromatography, (iv) $AgNO_3$ columns for chloride precipitation, or (v) ion precipitation with acetone.

While the SPE SCX and Oasis MAX columns both consistently retained basic and acidic compounds respectively, we found SAX columns to be less predictable, e.g., mycophenolic acid is not well retained on SAX (<10%) while moniliformin[60] and fumonisins are quantitatively retained.[21] Also, problems with loading capacity due to excessive amounts of organic acids in the crude extracts add to this unpredictability. We have observed this with *Aspergillus niger* extracts producing high levels of citric acid.

The original bioactivity of an extract can sometimes be lost upon fractionation. This may be due to instability under the conditions applied, e.g. acid/base, light, or reactive solvent, or it could be due to strong retention of the active compound on the selected column. For example, phosphates and sulphates will not be eluted by lowering pH on SAX/MAX columns but need salting out or the use of a weak anion-exchanger (WAX), while quaternary amines on SCX columns also need salting out or the use of a weak cation exchanger (WCX), e.g., a carboxylic acid (CBA) column.

A distribution analysis of calculated pK_a's from the 34390 records in Antibase2008 revealed that within pH 2−11, 44% of all the records had acidic, 17%, basic, and 9%, both functionalities. This shows great potential for using ion-exchange chromatography as an integral part of the separation procedure, which we pursued by E-SPE using SAX, Oasis MAX, SCX, and Sephadex LH-20 columns in a setup giving 15 fractions for biological evaluation. By employing orthogonal chromatographic methods, the E-SPE approach presents a consistent setup for accelerated discovery of novel compounds and offers faster dereplication and purification and uses fewer, more efficient steps. The setup can readily be tailor-made to suit individual laboratories with varying access to assays or equipment. We trust that the E-SPE protocol will encourage other NP groups to publish their methods and share their "tricks of the trade" with the community.

Experimental Section

General Experimental Procedures. Solvents and buffers were all HPLC or LC-MS grade. All aqueous solutions were prepared from H_2O obtained from a Milli-Q system from Millipore (Millerica, MA).

Samples were analyzed using an Agilent 1100 HPLC system with a diode array detector (Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. A Phenomenex (Phenomenex, Torrance, CA) Luna II C_{18} column (50 mm ×2 mm, 3 μm) was used for separation, applying an MeCN−H_2O 0.3 mL min^{-1} gradient (15−100%) over 20 min at 40 °C. Both MeCN (LC-MS grade) and H_2O were buffered with 20 mM formic acid (LC-MS grade).

Large-scale cation SPE purification of the *P. luteoviolacea* extract was performed on an Isolera (Biotage, Uppsala, Sweden) automated flash system. Details are given in the Supporting Information.

NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 MHz) equipped with a Protasis CapNMR capillary probe, using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 3.3 and δ_C 49.3 ppm for methanol-d_4).

Bioassays were performed using standard protocols.[61]

Theoretical pK_a calculations of Antibase2008 were made by converting a ChemFinder version of Antibase2008 to an sdf file using ChemFinder (Cambridgesoft, Cambridge, UK) and then importing the sdf file into ACD ChemFolder (Advanced Chemistry Development, ACD, Toronto, Canada) and then batch calculating the pK_a values using the ACD 2008 pK_a suite.

Biological Material. The marine strain included in the study, *Pseudoalteromonas luteoviolacea*, was selected due to its ability to antagonize the fish pathogenic bacteria, *Vibrio anguillarum*.[62] *P. luteociolaceum* was isolated from seaweed (latitude: 2.9817 N, longitude: −86.6892) and identified based on phenotypic tests such as Gram reaction, cell shape, motility, and glucose metabolism, as well as 16S rRNA gene sequence homology. The bacterium was routinely cultured in Marine Broth (Difco 2216). Three-day static cultures (25 °C) were used for extractions.

The fungus used was from the IBT collection at the Center for Microbial Biotechnology, Denmark, and authenticated by Prof. J. C. Frisvad. *Penicillium roqueforti* (IBT 16404) was grown on one plate of CYA agar for 7 days (25 °C, dark).

Sample Preparation. Bacterial cultures (25 mL) were freeze-dried and extracted EtOH:H_2O (96:4 v/v, 2 × 10 mL) for partial desalting (no sonication; 2 × 12 h), filtered, pooled, and evaporated to dryness with N_2 flow. The agar plate containing the fungal culture was homogenized using a stomacher and extracted directly with 20 mL CH_2Cl_2:EtOAc:MeOH (3:2:1 v/v/v) and then a 50:50 (v/v) mixture of MeCN:H_2O (20 mL). Extracts were filtered, pooled, and evaporated to dryness with N_2 flow.

E-SPE Procedures. All extracts were redissolved in 400 μL of the loading solution prior to running each column, except for Sephadex LH-20 where 100 μL of MeOH was used.

The strong anion-exchange step was performed using Strata-SAX columns (Phenomenex, 100 mg/1 mL), using MeOH:H_2O (70%) for extract loading and wash, and MeOH (1% formic acid or 0.5 M K_3PO_4) for elution, collecting two fractions (A1 = unretained and A2 = retained).

Mixed-mode reverse-phase anion-exchange was performed on an Oasis MAX column (Waters, Milford, MA, 30 mg/1 mL, 30 μm). A MeOH:H_2O solution (25%) with NH_4OH (2%) was used to lock and load the column. A series of MeOH:H_2O solutions (25%, 60%, and 100%) were used for eluting nonacidic compounds, and an equal series of MeOH:H_2O solutions (25%, 60%, and 100%) acidified with formic acid (1%) for eluting acidic compounds. For the MAX column, a total of six fractions were collected, i.e., B1, B2, and B3 = unretained polar/25% MeOH, medium polar/60% MeOH, and apolar/100% MeOH; B4, B5, and B6 = retained polar/25% MeOH + 1% formic acid, medium polar/60% MeOH + 1% formic acid, and apolar/100% MeOH + 1% formic acid.

The strong cation-exchange was performed on Strata SCX (Phenomenex, 100 mg/1 mL, 33 μm) using MeOH:H_2O (70%) for loading and wash, and MeOH with NH_4OH (2%) for elution, collecting two fractions (C1 = unretained and C2 = retained).

For size exclusion, Sephadex LH-20 (GE Healthcare, Hillerød, Denmark) was swelled in MeOH and wet packed in syringes (1 mL) (25 mg/1 mL, 27−163 μm) equipped with a frit, top and bottom. For each extract (100 μL) a total of five fractions were collected, either band-based collection for colored extracts or time-based (1:0.5:1:2:4 mL), respectively.

For all four columns, a blank medium sample corresponding to the media used for cultivation was subjected to the same fractionation and represented a negative control.

Detailed protocol can be found in the Supporting Information.

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Supporting Information Available: Experimental details on the purification of indolmycin and violacein, and the E-SPE protocol is available free of charge via the Internet at http://pubs.acs.org.

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Supplementary information E-SPE

Purification of indolmycin and violacein. The bacterial broth (8 L) was extracted with a mixture (50/50) of sterile XAD 7 (Sigma-Aldrich, St. Louis, MO) and HP20 resin (Sigma-Aldrich, St. Louis, MO) (12 g of resin/L broth). After 24 h the resin was filtered off and washed with water (2 x 2 L), followed by extraction with methanol/water (50/50 v/v; 800 mL) (24h) and methanol (2 x 800 mL) (24 h). All organic extracts were pooled and dried completely to give a crude extract (2.0712 g). The crude was re-dissolved in methanol and water (90/10 v/v) and mixed with 20 g Strata-XC (Phenomenex, Torrance, CA) and dried before packing into a 25 g SNAP column (Biotage, Uppsala, Sweden) with pure resin (5 g) in the base. The column was equilibrated (25 mL/min) upside-down with water + formic acid (1%). The column was then turned and washed with methanol with formic acid (1%) (fraction 1; 782 mg). The column was then stripped with methanol with ammonium hydroxide (2%) and washed until no colour remained (fraction 2; 1.0368 g). Fraction 2 was found to be enriched with indolmycin and violacein. These two compounds were purified on an LH20 column using methanol as eluent (80 g; 100 x 2 cm; 100 mg; 0.5 mL/min).

SOP for E-SPE:

1) Prepare vials and labels.

2) Prepare solvents:

100% water (pH 7)

25% MeOH (pH 7)

60% MeOH (pH 7)

70% MeOH (pH 7)

100% MeOH (pH 7)

25% MeOH (1% formic acid, pH 2)

60% MeOH (1% formic acid, pH 2)

100% MeOH (1% formic acid, pH 2)

100% MeOH (2% ammonium hydroxide, pH 11)

3) Prepare LH-20 columns:

A 1 mL syringe (75 x 5 mm) is fitted with a frit in the bottom and wet packed (in MeOH) until 0.9 mL corresponding to approximately 20-30 g dry LH-20. Frit added on top as well. If dry packed, let the LH-20 swell in the MeOH for 30 min. before use.

4) Prepare crude extracts:

a) *Bacterial extracts:*

25 mL of culture is freeze-dried (-80 °C for marine cultures) and extracted with 96% EtOH (2 x 10 mL) over 2 x 12 h. Centrifuge (8500 rcf/15 min) to separate cells and undissolved media components from extract. The extract is filtered (0.45 µm).

b) *Fungal extracts:*

One agar plate with the fungus is homogenized using a stomacher (1 min) and extracted over 2 x 12 h with 20 ml dichlormethane:ethyl acetate:methanol (3:2:1, v/v/v) and then a 50:50 (v/v) mixture of acetonitrile/water (20 mL). The extracts are pooled and filtered (0.45 µm).

5) Preparation of samples:

⅕ of the crude extract (4 mL for bacterial/8 mL for fungal) is taken directly for assay and 100 µL for LC-MS.

⅕ of the crude extract (4 mL for bacterial/8 mL for fungal) is taken for each of the four SPE columns (≈0.5-2 mg each).

The total of 5 vials are dried down.

6) Running the SPE columns:

a) *SAX (Strata SAX, 33 µm, 100 mg/1 mL):*

Sample is dissolved in 400 µL 70% MeOH (pH 7). Unless the sample has a high salt content, increase sample pH with 8 µL ammonium hydroxide (2%).

Column equilibrated with 2 x column volumes 100% MeOH followed by 70% MeOH in water (pH 7).

Sample load and wash with 1 mL 70% MeOH (pH 7) and 1 mL 100% MeOH (pH 7) = fraction A1.

Elute with 2 mL 100% MeOH (1% formic acid, pH 2) = fraction A2.

100 µL of each fraction taken for LC-MS analysis.

All fractions are dried completely.

b) *MAX (Oasis MAX, 30 µm, 30 mg/1 mL):*

Sample is dissolved in 400 µL 25% MeOH (pH 7). Unless the sample has a high salt content, increase sample pH with 8 µL ammonium hydroxide (2%).

Column equilibrated with 2 x column volumes 100% MeOH, followed by 2 x column volumes 100% water (2% ammonium hydroxide, pH 11).

Sample load and wash with 2 mL with 25% MeOH (pH 7) = fraction B1.

Elute with 2 mL 60% MeOH (pH 7) = fraction B2.

Elute with 2 mL 100% MeOH (pH 7) = fraction B3.

Elute with 1 mL 100% water (pH 7) and 1 mL 25% MeOH (1% formic acid, pH 2) = fraction B4.

Elute with 2 mL 60% MeOH (1% formic acid, pH 2) = fraction B5.

Elute with 2 mL 100% MeOH (1% formic acid, pH 2) = fraction B6.

100 µL of each fraction taken for LC-MS analysis.

All fractions are dried completely.

c) *SCX (Strata SCX, 33 um, 100 mg/1 mL):*

Sample dissolved in 400 µL 70% MeOH (pH 7). Unless the sample has a high salt content, lower sample pH with 4 µL formic acid (1%).

Column equilibrated with 2 x column volumes 100% MeOH, followed by 2 x column volumes 70% MeOH (pH 7).

Sample load and wash with 1 mL 70% MeOH (pH 7) and 1 mL 100% MeOH (pH 7) = fraction C1.

Elute with 2 mL 100% MeOH (2% ammonium hydroxide, pH 11) = fraction C2.

100 µL of each fraction taken for MS analysis.

All fractions are dried completely.

d) *LH-20 (self-packed, 20-30 mg/1 mL, 75 x 5 mm):*

Sample dissolved in 100 µL 100% MeOH.

Column equilibrated with 100% MeOH until constant flow from column (125-150 mL/min).

Load sample and let band focus for a short while before starting elution.

Band-based collection is preferable if extract is coloured.

For non-coloured extracts, collect:

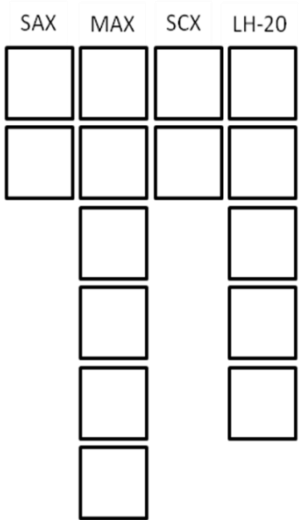
1 : 0.5 : 1 : 2 : 4 mL = D1 : D2 : D3 : D4 : D5

100 µL of each fraction taken for MS analysis.

All fractions are dried completely.

7) Prepare for assays:

The dry samples, including blanks and crude extract, are re-dissolved in 500 µL 50/50 (v/v) acetonitrile in water or another solvent compatible with the given assay. Assay-results are put into a bioactivity matrix:



Paper 2

"Dereplication of Microbial Natural Products by LC-DAD-TOFMS: Experiences Gained from an Inhouse Database of 718 Mycotoxins and other Microbial Metabolites"

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Dereplication of microbial natural products by LC-DAD-TOFMS: Experiences gained from an inhouse database of 718 mycotoxins and microbial metabolites

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Dereplication has become a key element of modern NP chemistry. The constant development of analytical equipment and compound databases has made it easier to determine the elemental composition, and thus tentative identification of a given compound in a sample. A major challenge remain to establish correct molecular formulas using mass spectrometry due to the diverse and complex nature of NPs such as polyketides, alkaloids, and terpenoids. Herein, we demonstrate the scope of LC-MS dereplication using data from UV/VIS DAD and ESI^+/ESI^- electrospray ionization on a Time-of-Flight MS for assignment of our 718 natural product standards. ESI^+ proved to be the most versatile ionization method, allowing detection of 93% of the compounds including 12% that ionized poorly. Simultaneous detection of the $[M+H]^+$ and $[M-H]^-$ ion was observed for 60% of the compounds, but only for 37% they were both the most intense ions. From ESI^+ alone, 56% of the compounds could be unambiguously assigned based on multiple adduct ions. From ESI^-, this number was 37%, however with further 41% producing only $[M-H]^-$. The polarity where the most intense ionization was observed was always the most trustworthy for interepretation. Poor ionization was more prevalent for small molecules (<200 Da). In ESI^-, non-ionizing molecules were often polar basic compounds, and in ESI^+ small aromatic acids or anthraquinones. No single ion-source settings could be applied over m/z 60-2000 range, thus it was necessary to continuously switch between three settings during the chromatographic run to allow both small labile molecules and large peptides into the MS, at the same time providing pseudo MS/MS. (258 words)

Microorganisms constitute an immense resource of natural product diversity that is candidates for drug development, food and feed additives, and other industrial products *(1-6)*. Dereplication *(7-9)* of already known compounds and their potential analogues is a vital part of the discovery process in order to minimize time and resource consumption *(1, 10-13)*. Several approaches to the dereplication process exist, typically based on a combination of chromatographic and spectroscopic methods and database searching. The largest and most specialized database for microbial natural products is *Antibase*, constructed and maintained by Laatsch and co-workers *(48)* and originally based on a substantial inhouse database.

Over the last 10-15 years, liquid chromatography-mass spectrometry (LC-MS) has gradually replaced LC-UV/VIS diode array detection (DAD) as the preferred dereplication method for small molecules [ref]. This is largely due to the introduction of atmospheric pressure ionization (API) techniques like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) *(9, 14-20)*, which allows a more gentle and versatile ionization of compounds. Also, dereplication by LC-MS has the major advantage that the accurate mass of compounds can be used as query in nearly all natural products databases. Even though dereplication by LC-NMR has had a major breakthrough within the last few years owe to the development in nano- and cryoprobes [molinski09, lang08] *(21)*, LC-MS is still far more sensitive, providing reliable results within the nanogam range *(10, 22, 23)*.

MS instruments are continuously being improved, currently making it possible to obtain both positive and negative ionization spectra even during fast ultra-high performance liquid chromatography (UHPLC). Quadrupole-time-of-flight (QTOF) and ion-trap instruments are capable of making data-dependent MS/MS on all major peaks without the need to predefine masses, augmenting the data obtained for dereplication in a single LC run. Moreover, TOF, orbitrap, and

63 fourier transform ion cyclotron resonance (FT-ICR) instruments provide mass accuracies in the 0.5-
64 1 ppm range, leading to only one or very few possible elementary compositions for a given ion peak
65 *(9, 14-20)*. However, at this writing the best accurate mass instruments cannot make
66 positive/negative switching fast enough for the ultra narrow peaks obtained by UHPLC.

67 Dereplication of unknown compounds relies on accurate mass, leading to a minimum
68 of possible elementary compositions to be used as query in a database search with only a few
69 candidates *(24)*. Recently, Kind et al. *(25)* found that compounds within a 0.5-5 ppm mass range
70 often have very different number of carbon atoms, demonstrating that mass accuracy and isotope
71 ratio are almost orthogonal parameters that can be used for excluding hypothetical elementary
72 compositions. Integration of mass accuracy and isotope model fitting into one weighted parameter
73 is consequently incorporated into most of the major MS vendors software packages, e.g. 'SigmaFit'
74 (Bruker Daltonics), 'I-fit' (MassLynx, Waters), and 'Score'(Mass Hunter, Agilent Technologies).

75 For compounds classes biosynthesized from units with same elementary composition
76 such as peptides, rhamnolipids, or polysaccharides, accurate mass compared to nominal mass
77 determination provide limited information. Here MS/MS, or even better MS^n, and the subsequent
78 fragmentation patterns is more efficient, assuming that reference standards are available for
79 modeling fragmentation of the compounds class *(26, 27)*.

80 Despite many indisputable advantages, LC-MS screening with ESI or APCI faces five
81 major problems: i) sensitivity is compound dependent with some being unable to ionize in positive
82 and/or negative polarity, which can lead to incorrect assignment of ions to co-eluting impurities as
83 well as ion suppresion; ii) the adduct pattern $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M-H]^-$, $[M+HCOO]^-$,
84 etc. supporting correct assignment of the molecular ion can be difficult to interpret *(21, 22, 28, 29)*;

iii) the adduct pattern varies substantially from system to system, and can even change during a sequence due to sodium extraction from solvent glass bottles *(22, 30-33)*; iv) some compounds form predominant di- and trimeric ions e.g. $[2M+H]^+$, $[2M+Na]^+$, $[3M+H]^+$, and $[2M-H]^-$, which can further complicate assignment; and finally v) some compounds fragment very easily, losing one or more H_2O equivalents, formic acid, acetic acid and/or CO_2 *(34)*. Either of these problems can result in an erroneous assignment of the molecular mass *(22, 29)*.

Several studies focusing on adduct formation can be found in the literature *(22, 28, 32, 33)*, adding up to approximately 20 charged adducts and simple fragments from ESI^+ (including $[M+H]^+$ and doubly charged ions) and about 11 different from ESI^- (including $[M-H]^-$ and doubly charged ions). All studies were performed within a single compound class such as trichothecenes *(35)* and sugars *(36)*, and to the best of our knowledge, no large survey exists on adduct formation between different classes of compounds.

Dereplication can be an arduous task, especially when candidates with similar masses from a databa se hit list need to be evaluated. Here UV/VIS *(22)* and MS/MS *(31)* data can be used along with taxonomic knowledge of the producing organism and its close relatives *(22)*. Taxonomy is far more discriminatory for eukaryotes, e.g. few secondary metabolites are found across several fungal genera*(41)*[larsen05], than for bacteria due to less frequent horizontal gene-transfer *(42)*. Candidates can also be differentiated based on the presence of ionizable groups by explorative solid-phase extraction (E-SPE) *(43)*. This approach can be used to separate neutral, basic, acidic, and amphoteric compounds as well as to unmask co-eluting compounds and thereby simplify subsequent LC-MS spectra of individual fractions for analysis. Other parameters that can be used for elimination of candidates include chromatographic retention. For neutral compounds, the calculated octanol-water partition coefficient cLogP and its pH corrected LogD correlate with

retention time *(44-46)*. This was demonstrated for acylhomoserine lactones where a convincing R^2 = 0.993 was found between RT and LogD *(47)*.

The objective of this study was to obtain a detailed understanding of adduct and fragmentation formation in ESI^+ and ESI^- for a database of 718 known compounds and to highlight compounds classes not easily detected by ESI^+ and ESI^-. This has not been done in a large scale for natural products, aside from our last study from 2003 *(22)* investigating ESI^+ spectra of 474 compounds. We also evaluate the impact of mass and isotope pattern accuracies for differentiating elementary compositions of 33,136 valid compounds in Antibase2008. Finally we have explored the accuracy of LogD calculated via ACD for predicting retention times in order to eliminate or verify candidates during dereplication.

Results and Discussion

[the structure of this section is not yet adjusted for Journal of Natural Products. Different topics will be discussed on the following sub-headings]

Antibase and mass accuracy. To characterize the general mass distribution of microbial natural products, the accurate mass was calculated for all compounds in AntiBase2008 *(48)*. Antibase, which is used extensively in our laboratory, contains data for secondary metabolites from microorganisms and higher fungi, including yeasts, ascomycetes, basidiomycetes, and lichens, and also algae and cyanobacteria *(48)*. For important fungal genera such as *Aspergillus, Penicillium, Alternaria, Stachybotrys,* and *Fusarium,* we found AntiBase to contain as much as 95-98% of the compounds published in journal papers and patents. Before investigating the data in AntiBase the approximately 34,300 records were and stripped for: i) compounds with monoisotopic masses below 60 Da; ii) compounds not containing hydrogen; iii) and $C_{24}H_{38}N_{12}O_{12}$ (used in the database for unidentified peptides), resulting in 33,136 valid records.

In table 1, the number of elementary compositions differing by less than 10, 5, 2, and 1 ppm, respectively, is divided into mass intervals of 100 Da. For example, within mass range 500-599 Da there are 132 compounds that cannot be differentiated with a mass difference of 5 ppm while 58 cannot at 2 ppm mass difference. The number of candidates can be reduced by 25-50% when isotope ratios are used such as the number carbon atoms (A+1 corresponding to $^{12}C/^{13}C$ ratio) and halogens or sulfur (A+2 corresponding to $^{79}Br/^{81}Br$, $^{35}Cl/^{37}Cl$, or $^{32}S/^{34}S$). Sulfur can be difficult to detect due to low natural abundance of the heavier isotope, nonetheless it is found in 6.9% of the records and thus relevant for dereplication purposes. Another way to recognize sulfur containing compounds is by a low mass defect caused by the very high negative mass defect of sulfur itself

(monoisotopic mass 31.9721). The newest TOF generations from Agilent, Waters, Bruker, and Sciex are able to provide high isotope ratio accuracy of 1-5 %. The distribution found in AntiBase2008 confirms the results of Kind et al. *(25)* on hypothetically generated compositions within mass range 0-500 Da.

The observed mass distribution of NPs within AntiBase2008 clearly stresses the importance and need for high mass accuracy in a high throughput dereplication setup: With less than 1 ppm mass accuracy, there are no overlapping candidates in AntiBase2008 (**Table 1**) even without information on isotopes. However, in practice the mass accuracy of the MS instrument has to be two- to four-fold better in order to compensate for fluctuations in the actual measurements to make sure two compositions are not confused *(28).* Such a level of accuracy can currently be achieved on FT-ICR, Orbitrap, and the newest TOF instruments if internal mass correction is used.

Figure 1 presents the number of compounds with the same elemental composition as a function of the monoisotopic mass in AntiBase2008. Interestingly, the compounds with the highest redundancy in composition were all terpenes, which makes sense due to the numerous variations that exists of e.g. non oxygenated mono- ($C_{10}H_{16}$) and sesquiterpenoids ($C_{15}H_{24}$) in fungi, due to the incorporation of repetitive isoprene units into these compounds. For most such compounds, proper isotope ratio, retention times (RT), MS/MS data, and/or prerequisite knowledge of microbial origin is imperative for successful dereplication. This is very time consuming, especially for compositions like $C_{15}H_{22}O_3$ where 113 candidates exist in Antibase2008. Thus, there is a need for *in-silico* tools for automated fragmentation analysis of especially compounds with same molecular mass *(49).*

Statistical analysis based on 718 metabolites. In order to search for compounds in databases, the mass or molecular formula must be established unambiguously. Defining the molecular ion is far from evident based on ESI generated data, and knowledge of the adduct pattern is paramount. With the purpose of developing a general strategy for deducing ESI spectra, we compiled data from all 718 compounds in our in-house collection and stored these in an ACD database *(43)*. Each entry contained chemical structure, chromatographically validated (deconvoluted) monoisotopic ions from ESI^+ and ESI^-, UV/VIS spectrum (pH 3-3.3), UV absorption maxima, and retention index (retention time relative to alkylphenones) *(50)*. Also, ions corresponding to more than 5% relative to the most intense ion in ESI^+ and ESI^- ions from three different scan-functions were compiled. The data can be seen in Table 2 (and as Supporting Information, Excel format). As double charged ions were rarely observed and their formation dependent on ion-source settings *(51)*, they were not included in Table 2. Paracelsins was the only compound class where double charged ions were observed regularly, and all are in the 1900-2000 Da range, which is in the absolute upper-range of secondary metabolites (**Figure 1**). Likewise, we did not include dimeric $[2M+X]^+$ and trimeric ions $[3M+X]^+$ *(28, 33)* since the intensity of these was highly irreproducible due to concentration-dependent formation *(51)* (**Figure 3**).

The most common adducts and fragments observed from the ESI^+ and ESI^- spectral data of the 718 compounds in the database are noted in Table 3. Also, the frequency at which they occur is noted in the table (see also supplementary for further data). Adducts and fragments are recognized based on their relative mass (Δ) compared to $[M+H]^+$ or $[M-H]^-$, creating a characteristic pattern of ions. Figure 4 illustrates the annotation of Δ-values in ESI^+ and ESI^- spectra of asperphenemate to gain correct mass assignment.

In ESI^+, the molecular ion $[M+H]^+$ is in most cases the predominant ion observed in the spectrum, and the ion is formed from 88.5% of the molecules (see **Table 3**). 27% of the compounds produced $[M+H]^+$ as the only ion. It leaves doubts to if it is $[M+H]^+$, $[M+H-H_2O]^+$, $[M+Na]^+$, or $[M+NH_4]^+$ ion, however this can usually be revealed by careful comparison of the different scan functions. Also, most compounds making $[M+Na]^+$ or $[M+NH_4]^+$ usually make both, causing the characteristic $\Delta 5$, which results in <3% risk of assigning $[M+H]^+$ incorrectly. This fraction is lower than for drug molecules *(52, 53)* since most of these contain amines added for enhanced solubility and bioavailability. In contrast, the number of fungal and bacterial secondary metabolites with amine functionalities constitutes 8% and 28%, respectively *(43)*.

The acetonitrile adduct is the most frequently formed adduct in ESI^+, when using acetonitrile-water as the mobile phase. Small molecules (<250 Da) like pyrones often display very prominent $[M+H+MeCN]^+$ ions (*m/z* $\Delta 41$), often accompanied by a strong $[M+Na+MeCN]^+$ ion (*m/z* $\Delta 63$) and in a few cases $[M+H-H_20+MeCN]^+$ (*m/z* $\Delta 23$). Interestingly, the same compounds did not ionize very well in negative mode. Due to its strong appearance, $[M+H+MeCN]^+$ can easily be mistaken for $[M+H]^+$, and thus special attention should be paid to the characteristic $\Delta 41$. However, there is however a risk to confuse the pair of $[M+H+MeCN]^+$ and $[M+Na+MeCN]^+$ with $[M+H]^+$ and $[M+Na]^+$. In most cases, increasing the skimmer potential resulted in lower abundance of $[M+H+MeCN]^+$, but for a few compounds it increased as if the MeCN attached to $[M+H]^+$ upon collision. In a few cases fragment ion with MeCN adducts were also observed.

A strong adduct with sodium $[M+Na]^+$ (*m/z* $\Delta 22$) was formed for 21.4% of the compounds in ESI^+, yet only in a few cases forming the most prominent ion. $[M+Na]^+$ was especially abundant for acids where it can ion-exchange on the carboxylic acid group to make $[M+2Na]^+$. In alkaloids, the abundance of $[M+Na]^+$ were usually very low, presumably due to a low

affinity for Na^+ compared to H^+ to the lone pair on the nitrogen. We speculate that the amines in many cases are protonated in the solvent state prior to leaving the droplets.

An ammonium adduct $[M+NH_4]^+$ (*m/z* Δ17) is observed for 19.3% of the compounds. The affinity of ammonium is especially high for oxygenated molecules, such as polyketides, where this ion is often the most predominant in the spectrum. In contrast, this adduct is extremely rare for alkaloids. For some primary amides and amino acids, a loss of NH_3 was observed $[M+H-NH_3]^+$, giving rise to the same mass difference of Δ17. However, as the $[M+NH_4]^+$ ion is often accompanied by $[M+Na]^+$, this set of ions can be identified by a characteristic mass difference of Δ5. The high abundance of $[M+NH_4]^+$ adducts was a surprise since ammonia was not added to any of the solvents. The intensity of $[M+NH_4]^+$ ion peaks usually increased during a sequence suggesting that NH_3 is formed as a result of acidic hydrolysis of MeCN. This was confirmed by using pure non-acidified MeCN. Substitution of MeCN with MeOH had an even stronger effect and almost eradicated ammonium adducts.

Not surprisingly, loss of water $[M+H-H_2O]^+$ (*m/z* Δ18) is the most common fragment ion formed. Inspection of the structures revealed that almost all lose H_2O from an alcohol group where a β-hydrogen is available for elimination.

Altogether ESI^+ was able to detect 93% of the compounds (**Table 4**), however including 12.4% poorly ionizing compounds. The compounds not able to ionize were mainly small acids with the acidic carbon positioned next to a conjugated system. In part, this removes the free lone pair of the oxygen so it is unavailable for H^+ binding. Anthraquinones are examples of such molecules where the most problems were encountered. The seven fold increase in non-ionizing compounds compared with our last study *(22)* was predominantly caused by an increase in this group of compounds in our

231 database. We found that the fraction of poorly ionizing and non-ionizing compounds could be
232 shifted with impurity levels in the solvents and how often the solvent bottles were changed. Besides
233 contamination of the ion-source with organic material, we observed that corrosion in the column
234 oven (observed as corrosion at the inlet) and ions liberated here from caused a 10-100 fold drop in
235 sensitivity for poorly ionizing compounds. Strongly ionizing compounds like alkaloids did not
236 suffer significantly from these issues. We observed a Na^+ efflux from the solvent bottles which
237 lowers sensitivity and cause ion-pair effects during HILIC separations for example *(54)*. This
238 problem can be met by substituting glass bottles for the water with special plastic bottles (we used
239 bottles from Dionex, Sunnyvale, CA).

240 Based on ESI^+ alone, only 56% of the compounds in our database could be
241 unambiguously assigned (**Table 4**), meaning that at least two adducts points towards the same
242 mass. In further 30% of the cases, only the $[M+H]^+$ ion was detected.

243 As previously observed *(33)*, ESI^- produced less adducts than ESI^+ (**Table 3**) and in
244 consequence, the risk of incorrect adduct assignment is 4.5% higher (supplementary material). The
245 most commonly formed adduct was formate adduct $[M+HCOO]^-$ (*m/z* Δ46). Loss of 46 was
246 fortunately rare (0.5%), thus Δ46 is therefore almost guaranteed a formate adduct. We speculate,
247 that formate in some instances is used to deliver the charge by forming $[M-HCOO]^-$, and leaving
248 $[M-H]^-$ by evaporation of formic acid. The formate adduct can often be confirmed by the presence
249 of a chlorine adduct $[M+Cl]^-$, and relative mass difference of Δ10 (and a Cl isotope pattern) is
250 therefore characteristic of this pair. In this study, $[M+HCOO]^-$ and $[M+Cl]^-$ were mainly formed on
251 neutral compounds why the detection of these adducts are therefore strong indicators of molecules
252 not containing carboxylic acids or acidic phenols.

253 Loss of CO_2 was the most common (14%) fragment ion formed for ESI^-. Among carboxylic acids,
254 37% lost CO_2 which was a 2½ fold higher prevalence than in the whole dataset, but still lower than
255 expected *(34, 55)*. Of the compounds losing CO_2 in negative mode, one third lost formic acid in
256 positive mode (5% in the whole dataset). Interestingly, loss of H_2O in negative mode was very rare
257 (**Table 3**) and only observed from 2% of the molecules investigated.

258 Metal ion adducts were interestingly also observed in negative mode with $[M-2H+Na]^-$
259 and $[M-H+HCOO+Na]^-$, which fitted well with ion-exchange processes. It was observed
260 for 4.5 % of all compounds investigated, and usually for compounds containing a carboxylic acid or
261 another acidic functionality. These adducts are also known from phospholipids *(56)*. Thus, $[M-2H+Na]^-$
262 is a strong indication of an acidic functionality in an unknown compound being
263 dereplicated. The positive adduct $[M+2Na-H]^+$ was occasionally observed but usually below 2%
264 abundance compared to the base peak.

265 Notably, 17 compounds including citrinin, oosporein, and mitorubrins produced $[M-H+H_2O]^-$
266 and $[M+H]^+$, respectively. They were in all cases highly conjugated compounds which
267 may be reduced and/or hydrolyzed during ESI^- and/or chromatography. On-column and in-source
268 reactions can be differentiated through observation of the chromatographic peak shape as one would
269 expect a non-Gaussian peak shape in the case of on-column reactions.

270 For only 36.5% of the compounds, the molecular mass could be unambiguously
271 assigned directly from ESI^- (**Table 4**). In the cases where a single ion is observed, it is most likely
272 to be $[M-H]^-$ (42.6%), and only in 7.2% of the cases could it be missassigned as $[M+HCOO]^-$. This
273 can easy be clarified by substituting the acid in the solvent to acetic acid. The lower fraction of $[M-H]^-$
274 compared to $[M+H]^+$ is likely due to fundamental differences in ionization mechanism. In ESI^+,

all charges (H^+, Na^+, K^+, NH_4^+) are added to the native molecule due to the Fe^{++} being liberated from from the steel capillary *(57, 58)*. In negative mode, $[M-H]^-$ is formed through loss of H^+, whereas all other ions in both polarities are formed by addition of a charged adduct .

One third of the compounds in our database did not ionize or ionized poorly in ESI^- (**Table 4**), thus making ESI^+ the first choice of ionization mode, unless *a priori* knowledge on the target compound(s) indicates ESI^-. It has to be noted that high concentration of formic acid suppress ionization in ESI^- due to low pH (more difficult to deprotonate) and competition with formate ions. We have been able to obtain better ESI^- sensitivity by reducing the concentration; however, to obtain the best possible peak shape and consistent retentions times, we choose to maintain 20 mM formic acid during chromatography.

A comparison of compounds producing $[M+H]^+$ and/or $[M-H]^-$ ions is shown in Table 4. Both ions can be observed for 60% of the compounds investigated, yet only in 37.4% of the cases do they represent the most abundant ion. As mentioned previously, 56.4% and 36.5 % of molecular masses could be assigned unambiguously using ESI^+ and ESI^-, respectively. The combination the two ionization modes, resulted in 93% correctly assigned masses. The remaining 7 % consisted of poorly/non-ionizing compounds, or compounds only ionizing strongly in one mode. For example, highly polar alkaloids like roquefortines A and B, as well as simple ergots alkaloids will ionize strong as $[M+H]^+$, but not at all show up in negative mode. Poorly ionization compounds were often small molecules (<200 Da) often with a structure appearing to be volatile (few polar groups and no charge at pH 3-3.3) and we find it likely that they are lost as neutrals with the nitrogen due to the high source temperature (350 °C) and presumable requires APCI ionization. It was clear that ionization efficiency increased during the gradient run due to the higher concentration of MeCN. This phenomenon was already observed in the early days of ESI *(59, 60)*. The sensitivity of poorly

ionizing polar compounds can therefore be enhanced by using improved columns for polar analytes such as pentafluorophenyl or HILIC columns *(61, 62)*. Perhaps the most important observation when comparing ESI^+ and ESI^- was that the polarity providing the most intense ionization always resulted in the most simple spectrum for interpretation and most reliable adduct pattern.

The frequencies of ionization as well as adduct patterns in ESI^+/ESI^- were compared to those of $ACPI^+/APCI^-$ on a small subset of 60 chemically diverse compounds (supplementary data), including some of the problematic compounds. This was done on an Agilent LC/MSD VL single quadrupole system using the exact the same chromatographic conditions, and after adjusting the fragmentor voltage to give comparable fragmentation to that of the Z-spray source. This ion-source showed slightly better ionization of anthraquinones and apolar compounds, whereas the Z-spray remained superior in ionization of small polar substances. However, the data did not indicate that the overall number of detectable compounds would be different from that of ESI.

In-source fragmentation versus ion-transmission. [preliminary section] A conflict exists in the ion source, as no large m/z ions (> m/z 800) were observed at the low skimmer potential difference of 12-20 V which were needed to avoid fragmentation of small labile compounds. Part of this must originate from the higher velocity of low m/z ions between the skimmers (cones) as their velocity is higher (skimmer potential difference = $\frac{1}{2} \times m \times V^2$. Thus must an m/z 60 ion have an almost 6 time higher velocity between the skimmers than an m/z 2000 ion. The only choice is thus to use fast (compared to the chromatographic peak width) alternating skimmers potentials (illustrated in **Figure 4**) to obtain a broad m/z range of ions transference from the ESI source to the MS.

The use of alternating cone potentials had another advantage between adduct ions and fragment ions by enhancing fragment ions and adducts with the very stable alkali metal ions *(29, 63)*. A third advantage is that pseudo MS/MS data can be obtained *(64)* as also seen in Figure 5. The overall concept is marketed as MS^E in QTOF instruments from Waters *(65)*.

Chromatography. [preliminary section] Due to the inherent nature of reversed phase chromatography, it is tempting to link retention and LogD which has been shown to correlate well for acylhomoserine lactones (R^2 = 0.993) *(47)*. As shown in Figure 5A, a similar correlation was obtained for the fumonisins (containing five ionizable groups), but for the neutral aflatoxins and sterigmatocystins (**Figure 5B**) the correlation was less convincing (R^2 = 0.65) with an average predicted error of the retention index of 55 and a max error of 95. In figure 6, all 718 compounds are presented which did not prove satisfactory (R^2 = 0.38). Several groups were analyzed individually and compounds like acylhomoserine lactones correlated excellently (R^2 =0.99) under our conditions. In general were compounds with LogD below -1 were not retained, thus did a lower logD not impact the retention index. This shows that calculated LogD values are still of limited use for exclusion of candidates during a dereplication process unless sufficient related compounds are evaluable as reference standards to verify a given regression model. The general poor correlation seen in Figure 6, must be due to factors as inaccurate pK_a calculation, ionic interactions with the underlying silica, steric effects of molecules, and ion-paring effects with $HCOO^-$ and H_3O^+ *(66-70)*.

[Major dereplication example]

Conclusion. Thorough investigation of adduct formation of 718 natural products have demonstrated that ESI^+ was the most versatile method capable of detecting 93% of the compounds. Using the adduct pattern, 56% of the compounds could be unambiguously assigned based on ESI^+ alone, whereas 37% could be assigned be unambiguously assigned by ESI^- alone. Additional 41% produced $[M-H]^-$ without any validating ions. No perfect ion-source settings were found to cover the full range of compounds m/z 60-2000. Consequently, it was necessary to do ultra-fast alternation between two-three settings during the chromatographic run to allow both small labile molecules and large peptides into the MS. This also provided pseudo MS/MS data. When combining data from the ESI^+ and ESI^- the polarity where the most intense ionization was observed always provided both the simplest spectrum to interpret and most trustworthy adduct pattern.

Experimental Section

General Experimental Procedures. Solvents were HPLC grade and all other chemicals analytical grade unless otherwise stated. They were all obtained from Sigma-Aldrich (Steinheim, Germany). Specifically for the LC gradient system MeCN was Sigma 5485, and the formic acid Fluka 56302 (for LC-MS). Water was purified from a Milli-Q system (Millipore, Bedford, MA). Alkylphenone standards for RI determination were diluted in MeOH in concentrations of 2-3 mM as previously described *(22, 50)*.

Theoretical p*K*a calculations of Antibase2008 *(21, 48)* were made by converting the ChemFinder version to the ACD ChemFolder (Advanced Chemistry Development, ACD, Toronto, Canada) and then batch calculating the logD values at pH 2.7, 3.0 and 3.3 using the ACD 2008 PhysChem suite *(43)* by Dr. Shahriar Jahanbakht (Chemacad, Obernai, France).

Reference standards. Metabolite standards have been collected over the last 30 years *(22, 50, 72)*, either from commercial sources, as gifts from different research groups, or from our own projects and therefore only available in micro to milligram quantities, some only about 50% pure. About one third of the standards have been purchased from Sigma-Aldrich, Axxora (Bingham, UK), Cayman (Ann Arbor, Mi), TebuBio (Le-Perray-en-Yvelines, France), Biopure (Tulln, Austria), Calbiochem, (San Diego, Ca), and ICN (Irvine, Ca). Reference standards were generally taken out as powder using a Pasteur pipette (1-2 mm of powder in the tip), transferred to an auto-sampler vial, and unless other stated 1.5 ml MeCN added to the vial. In case of highly polar substances not soluble in MeCN a few drops of water was added or 2-propanol was used. The standard solutions were kept at –20°C.

LC-DAD-TOFMS. Tuning and calibration in ESI^+ was done as previously described *(22)*. In negative mode the MS was tuned to a resolution of ca. 5000 FWHM on $[M-H]^-$ of leucine enkephaline, and calibrated on a solution of PEG-di acids (average MW 200 and 600), polyalanine, 1,6-dihydroxybenzoic acid, and chloramphenicol and diluted in MeCN-water (1:1 v/v).

The LockSpray connected to a custom made system equipped with 0.5 L bottle to which a positive pressure of 2-3 bar N_2 was applied giving a constant flow of ca. 20 µl/min leucine enkenkephaline solution (0.1 µg/mL in MeCN-water-formic acid (50:50:0.1). The protonated and deprotonated molecular ion was used as lock mass in ESI^+ and ESI^- respectively.

All analysis was done on an Agilent 1100 LC system with a diode array detector (DAD) coupled to a LCT oaTOF mass spectrometer (Micromass, Manchester, UK), with Z-spray electrospray source (ESI) and a LockSpray probe. The system was controlled by the MassLynx 4.0 software. Separation was done at 40°C on a 50×2 mm i.d., 3 µm, Luna C_{18} II column (Phenomenex, Torrance, CA) equipped with a Security Guard pre column, using a linear water-MeCN gradient with a constant flow of 0.3 ml/min, starting with 15% MeCN-water going to 100% MeCN in 20 min, maintaining 100% MeCN for 5 min, before returning to the start conditions in 2 min and equilibrating for 5 min. Formic acid 20 mM was added to both solvents. The UV spectra was collected by a diode array detector every 0.4 sec from 200 to 700 nm with a resolution of 2 nm.

The source was kept at 120°C and desolvation temperature was 400°C. Sample cone was continuously flipped between 18, 30 and 50 V (scan functions 1, 2 and 3 respectively), each for 0.5 sec, with scan ranges of m/z 100-2000 for the last. The lock mass scan was performed every 6 s at a sample cone of 30 V. Capillary was held a 3000 V in ESI^+ and 2000 V in ESI^-, and the desolvation flow held at 400-450 (ESI^+) and 675-725 L/hr (ESI^-).

Data analysis. Data from 718 metabolites are collected in Table 2. The table lists the metabolite, formula, retention index (RI) calculated as described by Frisvad & Thrane *(50)*, if the peak was highly asymmetrically with is marked(*) on the RI, UV data.

ESI^+ and ESI^- data are each separated into: i) how well the compound ionizes; ii) ions representing adducts with M (H^+, NH_4^+, Na^+, K^+, Cl^-, MeCN, H_2O and formate as well as combination of these) and simple losses (H_2O, NH_3, acetic, CH_3- and formic acid, CO_2, formaldehyde); iii) and significant diagnostic fragments (usually from scan function 2 of 3). Isotope data are only included under the significant fragments in case of loss of Cl. All ions of the individual components were confirmed by manual mass de-convolution. The accurate mass measurements were calculated from spectra obtained in the front or tail of the peak to reduce influence of detector dead time by keeping the ion counts below 1000 counts.

Data management. MS and UV spectra data were stored in a custom made database made in the ACD v. 12 Chemfolder. The structures of most compounds were imported from Antibase 2007 or 2008 *(21, 48)*. Table 1 in supplementary data is an Excel version of the database. Log D at pH 2.7, 3.0 and 3.3 were kindly calculated as batch calculations in and added to the database. UV data were made by background subtraction and it was verified that it was not saturated.

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Supporting Information Available: The main table on data is available free of charge via the Internet at http://pubs.acs.org.

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List of figures and tables

Figure 1. Plot of the number of compounds in AntiBase2008 (n = 33,136) with identical elementary compositions as a function of the monoisotopic mass.

Figure 2. ESI+ spectrum of brevianamide B from tail (top) and apex (bottom) of the chromatographic peak. A higher prevalence of dimeric ions is observed in the peak apex.

Figure 3. Asperphenemate ESI spectra (low in-source fragmentation): **A** ESI^+ and **B** ESI^- with annotations, where the molecular mass of 506 can easily be determined ambiguously both polarities, e.g. by the Δ 22 in ESI+ and the Δ10 in ESI^-. [preliminary figure]

Figure 4. ESI+ spectra of culmorin from above high, medium and low in-source fragmentation (25 / 35 / 60 V). [preliminary figure]

Figure 5. Correlation between calculated LogD (pH 3.0) and retention index for two groups of mycotoxins: the fumonisins (A) and sterigmatocystins and aflatoxins (B).

Figure 6. Regression analysis of the retention time of 718 secondary metabolites (collected over one year) and cLogD at the pH used in the chromatographic run (for details refer to experimental section).

Table 1. Distribution of compounds in AntiBase2008 with different elementary compositions which cannot be distinguished at different mass-differences, carbon isotope ration, and A+2 elements (S, Cl, Br) determination.

Table 2. Compiled data from 718 secondary metabolites, including name, molecular formula, monoisotopic mass (MI), retention index (RI, retention time relative to alkylphenones), most intense ions observed in ESI^+ and ESI^-, and UV absorption maxima.

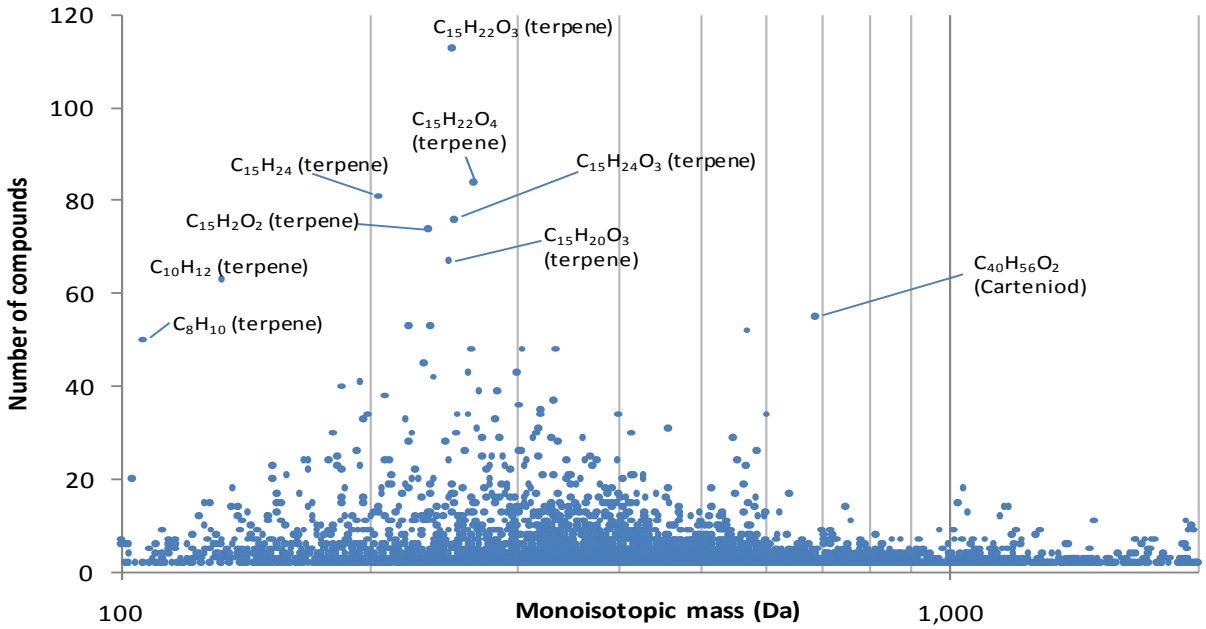
653 **Table 3.** Common adducts and fragments (Δ) observed in electrospray and the frequencies at which

654 they occur [data not added yet].

655 **Table 4.** Combined positive and negative ionization electrospray to determine correct molecular

656 mass.

657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80
60
40
20
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100
1,000
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C15H24 (terpene)
C15H24O3 (terpene)
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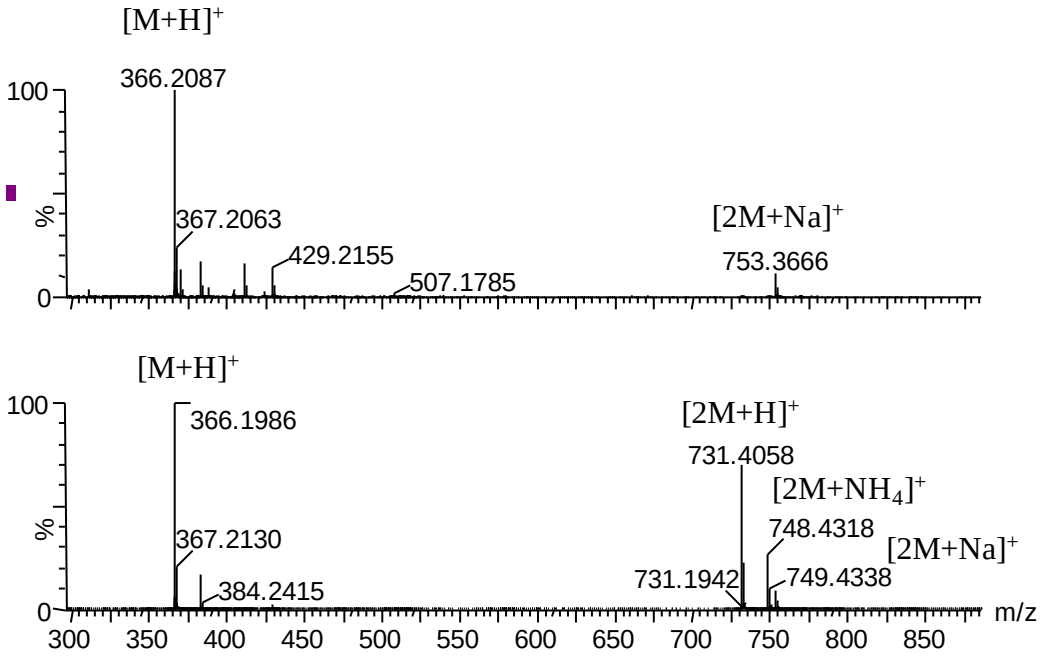
C40H56O2 (Carteniod)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100
%
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367.2063
429.2155
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[2M+Na]+
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[M+H]+
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[2M+H]+
731.4058
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748.4318
[2M+Na]+
367.2130
384.2415
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550
600
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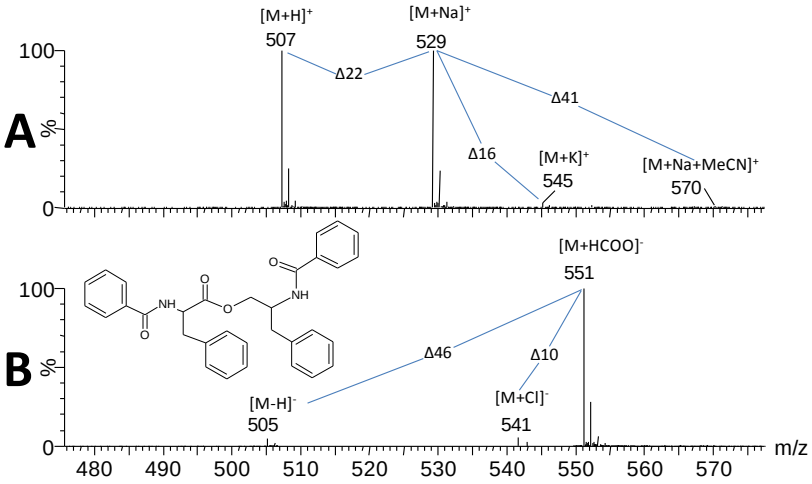
800
850

666

667

668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%
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m/z

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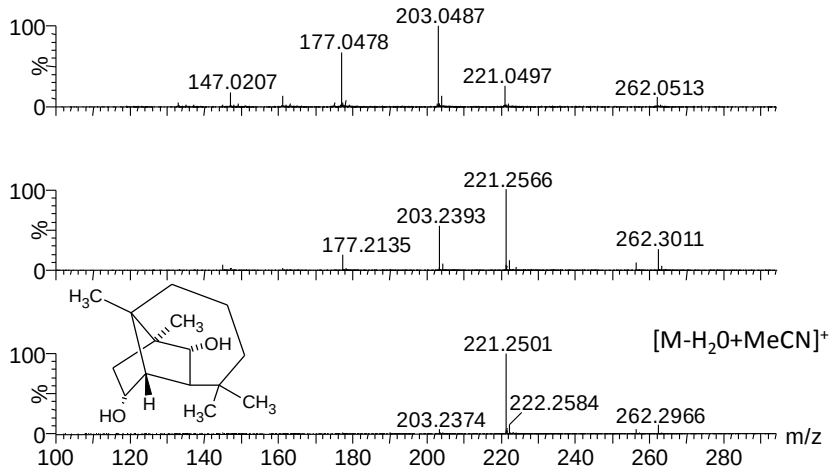
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HO
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H3C
CH3
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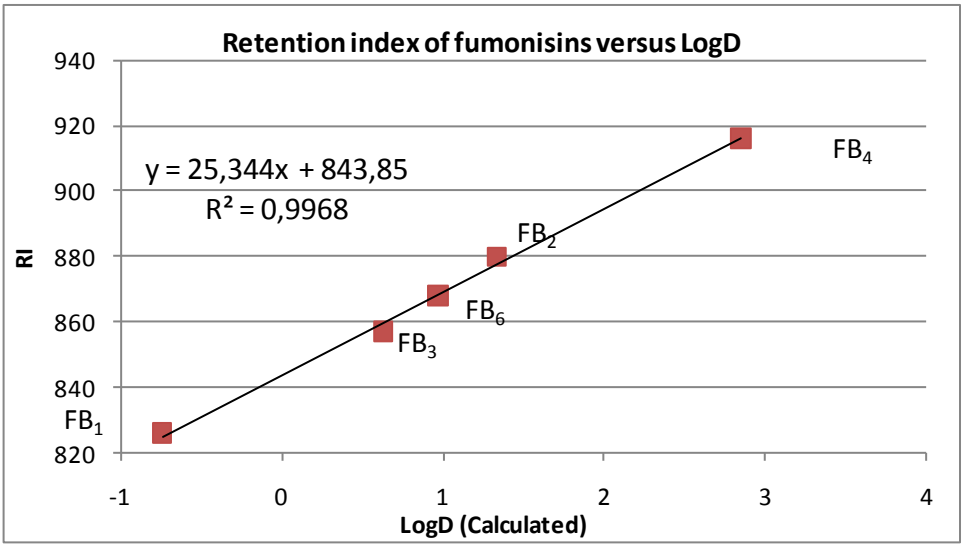
A



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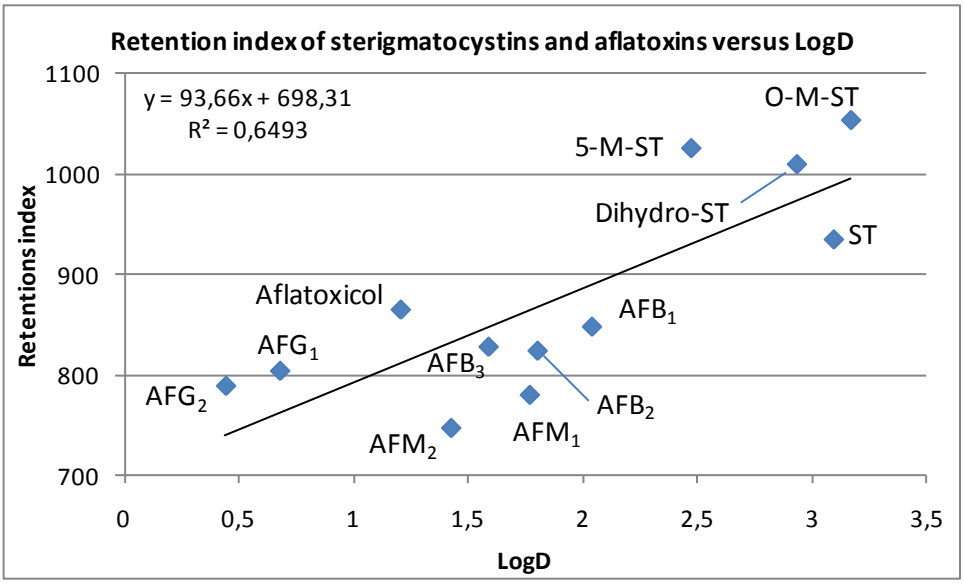

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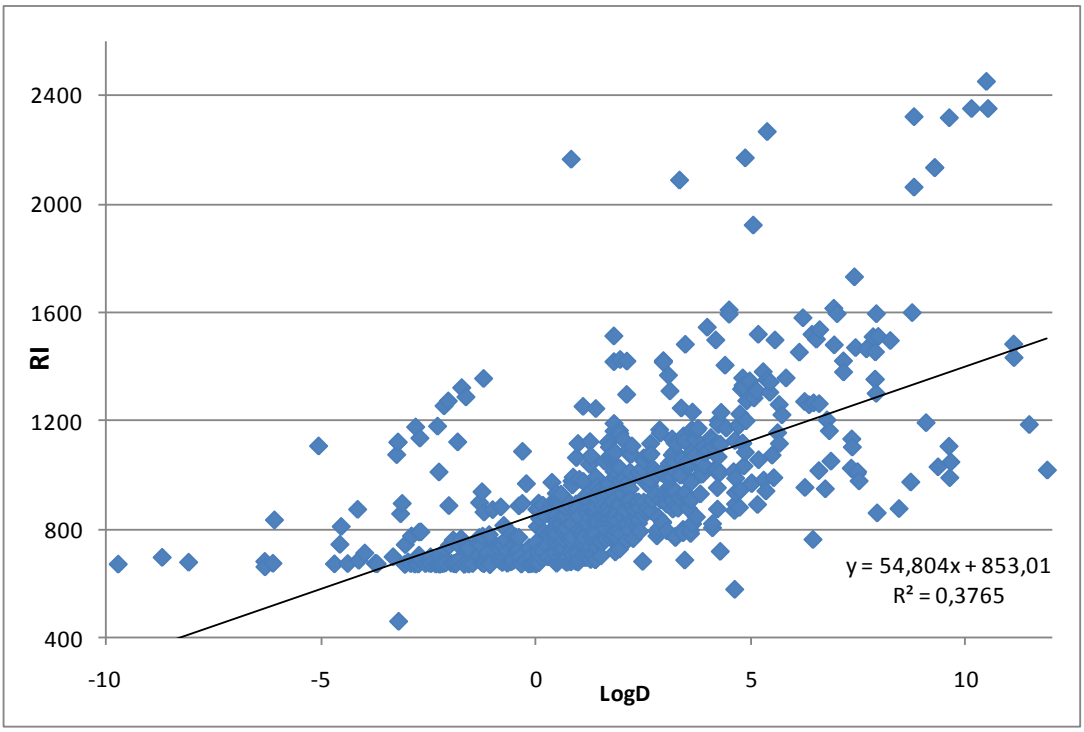
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1600
1200
800
400
RI
-10
-5
0
5
10
LogD
y = 54,804x + 853,01
R² = 0,3765

679

680

Distribution of compounds in Antibase 2008 with different elementary compositions which cannot be distinguished at different mass-differences[a], carbon isotope ratio, and A+2 elements (S, Cl, Br) determination

Mass range	Total compounds	Number of compounds															
(da)	Mass difference (ppm)	Nominal[b]				10	5	5	5	5	5	2	2	2	2	2	1
	Isotope constrain	None	±5 C	±2 C	±2 C[c]	None	None	±5 C	±2 C	±1 C	±1 C[c]	None	±5 C	±2 C	±1 C	±1 C[c]	None
60-99	133	45	45	38	31	0	0	0	0	0	0	0	0	0	0	0	0
100-199	2969	738	717	461	342	13	0	0	0	0	0	0	0	0	0	0	0
200-299	6167	1532	1376	811	538	104	48	43	40	32	19	0	0	0	0	0	0
300-399	6494	2119	1694	899	518	264	84	60	53	45	27	0	0	0	0	0	0
400-499	5125	2208	1636	872	567	434	195	163	138	114	92	0	0	0	0	0	0
500-599	3764	1791	1237	600	458	326	132	95	71	64	50	58	46	41	36	32	0
600-699	2065	1234	815	392	288	203	106	85	46	39	23	27	23	20	18	17	0
700-799	1590	985	669	352	273	145	69	55	26	25	23	23	20	16	16	16	0
800-899	1157	710	487	275	221	111	64	45	26	22	17	17	11	10	10	10	0
900-999	639	402	252	118	93	47	26	19	7	3	3	5	3	2	2	2	0
1000-3535	3033	1260	659	369	242	118	1	0	0	0	0	0	0	0	0	0	0
Total	33136	13024	9587	5187	3571	1775	730	1775	730	344	254	130	103	89	82	77	0

[a]Mass difference, thus meaning that 3-4 fold better instrument mass accuracy is needed to safely distinguish the two. [b] * mass defect of -0.3 to +0.7. [c]Assuming Cl, Br and S isotope information is being used

NAME	Formula	MI (amu)	RI	ESI+ data (m/z)	ESI- data (m/z)	UV_A
Oxalic acid	C2H2O4	89,9953	673	No ionization	89, 135 (PO)	End(100), 248(4)
Moniliformin	C4H2O3	98,0004	674	No ionization	97	228(100), 260(31)
1,3-Benzendiol	C6H6O2	110,0368	681	No ionization	155 (PO)	End(100), 216sh, 276(40)
Histamine	C5H9N3	111,0796	669	112 (PO)	156 (PO)	214
3-Nitropropionic acid	C3H5NO4	119,0219	680	No ionization	118, 164, 154	204
Benzoic acid	C7H6O2	122,0368	755	*164	121, 167, 189	228(60), 272(4)
Toluquinone	C7H6O2	122,0368	717	No ionization	No ionization	252(100), 325(7)
4-Ethylphenol	C8H10O	122,0732	867	No ionization	No ionization	200(100), 222(40), 278(12)
2-Phenylethanol	C8H10O	122,0732	755	No ionization	No ionization	End(100), 208(96), 260(4)
3,5-dihydrotoluen	C7H8O2	124,0524	690	125 (VPO)	No ionization	End(100), 220sh, 272(8)
Methyl-p-hydroquinone	C7H8O2	124,0524	686	No ionization	No ionization	End(100), 212sh, 290(25)
5-(hydroxymethyl)-2-furaldehyde	C6H6O3	126,0317	679	127, 144, 168 (PO)	No ionization	234(16), 286(100)
1,2,4-Benzenetriol	C6H6O3	126,0317	673	No ionization	171, 125, 161	End(100), 220sh, 290(14)
Maltol	C6H6O3	126,0317	678	168, 127	No ionization	216(100), 276(86)
methyl 2-furoate	C6H6O3	126,0317	778	No ionization	No ionization	254
Phloroglucin	C6H6O3	126,0317	673	127, 168, PO,	125, 171	204(100), 223sh, 270(4)
Pyrogallol	C6H6O3	126,0317	673	No ionization	125	207(100), 224sh(85), 264(46)
Cinnamic aldehyde	C9H8O	132,0575	832	174, 133 PO	No ionization	224(34), 297(100)
3-Aminobenzoic acid	C7H7NO2	137,0477	676	138, 179	136 (PO)	End(100), 220(96), 312(5)
4-Aminobenzoic acid	C7H7NO2	137,0477	683	179, 138	No ionization	End(100), 220(58), 286(97)
Anthranilic acid	C7H7NO2	137,0477	714	138, 179, 120	136, 182	218(100), 243(31), 330(16)
Salicylamide	C7H7NO2	137,0477	705	138, 179, 121, 162	136, 182, 172, 118	204(100), 236(32), 300(18)
Tyramine	C8H11NO	137,0841	663	179, 138, 121	136 (PO)	End(100), 220(86), 276(23)
1,3-benzodioxol-5-ol	C7H6O3	138,0317	726	No ionization	No ioniation	End(100), 233(15), 296(20)
2,3-dihydroxybenzaldehyde	C7H6O3	138,0317	714	No ionization	137 (VPO)	216(100), 268(72), 324(12)
2,4-dihydroxybenzaldehyde	C7H6O3	138,0317	725	139, 180, 275 (PO)	137	212(90), 230(65), 278(100), 312sh
2,5-dihydroxybenzaldehyde	C7H6O3	138,0317	706	275 (VPO)	137	230(100), 260(55), 360(21)
3,4-dihydroxybenzaldehyde	C7H6O3	138,0317	683	180 (PO)	137	214(100), 268(39), 328(12)
3,5-dihydroxybenzaldehyde	C7H6O3	138,0317	685	No ionization	137, 183, 173	214(100), 268(40), 328(10)
3-Hydroxybenzoic acid	C7H6O3	138,0317	697	No ionization	137	206 (100), 238(38), 296(11)
p-Hydroxybenzoic acid	C7H6O3	138,0317	689	No ionization	137, 183,	End(100), 208sh(85), 256(92)
Salicylic acid	C7H6O3	138,0317	770	No ionization	137, 183	204(100), 234(23), 300(11)
4-Hydroxyphenylethyl alcohol	C8H10O2	138,0681	687	121 (PO)	No ionization	End(100), 222(50), 276(11)
2,5-Dihydroxyanisol	C7H8O3	140,0473	680	141, 182 (PO)	185 (PO)	End(100), 254sh, 290(11)

3,5-Dihydroxyanisole	C7H8O3	140,0473	690	141, 182 (PO)	No ionization	205(100), 226sh, 270(5)
1,2-Dihydroxy-3-methoxybenzene	C7H8O3	140,0473	694	141 (VPO)	185, 139, 124	End(100), 226sh, 270(3)
3,5-Dihydroxybenzyl alcohol	C7H8O3	140,0473	675	141, 182, 164 PO	185, 139, 175, 113	202(100), 222sh, 278(11)
Butenolide	C6H7NO3	141,0426	675	183, 142	140, 186	End
Kojic acid	C6H6O4	142,0266	676	143, 184	141(VPO)	216(100), 268(78); (270, 7820)
Botryodiplodin	C7H12O3	144,0781	676	No ionization	143	210
Coumarin	C9H6O2	146,0368	778	188, 147	No ionization	End(100), 210sh, 278(54), 310(28)
Arabenoic acid	C6H10O4	146,0579	681	147, 188, 117	191, 145, 127 (PO)	240
Cinnamic acid	C9H8O2	148,0524	826	190, 131, 149	147, 193, 215	204(64), 216(65), 276broad(100)
5-Hydroxy-2(3H)-benzofuranone	C8H6O3	150,0317	697	No ionization	195, 149, 185, 121	End(100), 236(18), 290(11)
3-Phenylpropionic acid	C9H10O2	150,0681	812	192, 174, 146 (PO)	195, 149	End(100), 2908(82), 264(4)
2,6-Dihydroxyacetophenon	C8H8O3	152,0473	770	153, 194, 176	151, 197, 135, 133, 109	end(100), 222(80), 272(78), 344(14)
2,4-Dihydroxyacetophenon	C8H8O3	152,0473	756	153, 194, 135	151, 135, 109	210(100), 230(52), 276(90), 318(48)
2,5-Dihydroxyacetophenon	C8H8O3	152,0473	739	153, 194 (PO)	197, 151	236(100), 256(60), 358(29)
O-Vanilinin	C8H8O3	152,0473	766	153, 194	151 (VPO)	220(100), 266(70), 346(14)
3-hydroxy-4-methoxybenzaldehyde	C8H8O3	152,0473	711	153, 194	151, 136	208(100), 232(98), 280(82), 312(61)
3-hydroxy-4-methylbenzoic acid	C8H8O3	152,0473	703	194 (PO)	197, 151, 107	210(100), 246(44), 296(14)
3-Methylsalicylic acid	C8H8O3	152,0473	864	No ionization	151, 197, 107	208(100), 240(22), 308(11)
4-Methylsalicylic acid	C8H8O3	152,0473	836	No ionization	151, 107	208(100), 242(32), 300(13)
6-Methylsalicylic acid	C8H8O3	152,0473	800	194, 153, 135 (PO)	151, 107,	208(100), 244(22)sh, 302(10)
Gibepyrone F	C8H8O3	152,0473	701	194, 170, 153, 307, 324	No ionization	208(92), 248(18), 308(100)
p-Hydroxybenzoic acid methyl ester	C8H8O3	152,0473	763	194, 153, 121 (PO)	151, 197, 303, 136, 660, 631	End(100), 210sh, 258(84)
p-Hydroxyphenylacetic acid	C8H8O3	152,0473	687	No ionization	151, 197	End(100), 224(43), 276(9)
p-Hydroxyphenylacetic acid	C8H8O3	152,0473	689	194	151, 197,	End(100), 224(43), 276(9)
4-Ethylguaiacol	C9H12O2	152,0837	885	No ionization	No ionization	End(100), 226(19), 280(12)
2,3-dihydroxybenzoic acid	C7H6O4	154,0266	696	No ionization	153, 109, 169	206(100), 244(23), 310(14)
2,4-dihydroxybenzoic acid	C7H6O4	154,0266	697	196, 155, 178 (PO)	153, 109	208(100), 218sh, 254(41), 294(19)
2,5-Dihydroxybenzoic acid	C7H6O4	154,0266	692	No ionization	153, 109	208(100), 232sh(30), 324(18)

2,6-dihydroxybenzoic acid	C7H6O4	154,0266	744	No ionization	153, 135, 109	208(100), 246(31), 306(18)
3,4-dihydroxybenzoic acid	C7H6O4	154,0266	680	No ionization	153, 189, 109	206(100), 216(88), 260(50), 294(24)
3,5-dihydroxybenzoic acid	C7H6O4	154,0266	680	No ionization	199, 153, 109	208(100), 248(32), 306(13)
Patulin	C7H6O4	154,0266	677	196 (VPO)	153, 199	276
Terrein	C8H10O3	154,0630	680	No ionization	No ionization	282
3-Butyl-4-methylfuran-2(5H)-one	C9H14O2	154,0994	883	196, 155,	No ionization	220
Nonanoic acid	C9H18O2	158,1307	1059	No ionization	203, 157, 225	End
3-Indolylethanol	C10H11NO	161,0841	766	162, 203, 144	No ionization	222(100), 280(20)
p-Coumaric acid	C9H8O3	164,0473	714	206, 165, 147	163, 119	213(43), 227(51), 293sh, 308(100)
Phenylpyruvic acid	C9H8O3	164,0473	772	No ionization	No ionization	286
2,3,5,6-tetramethyl-1,4-benzoquinone	C10H12O2	164,0837	986	No ionization	No ionization	268
Gibepyrone A	C10H12O2	164,0837	902	165, 206	No ionization	End(89), 236(100), 332(92)
L-Phenylalanin	C9H11NO2	165,0790	673	207, 166, 120	164, 210, 232, 147	200(100), 210(98), 256(5)
α-Methoxyphenylacetic acid	C9H10O3	166,0630	731	208, 184, 176, 148	211, 165, 233	End(100), 208sh, 228sh
Ethyl-p-hydroxybenzoesyre	C9H10O3	166,0630	824	208, 180, 167, 162, 139, 121	165, 137	End(100), 258(80)
3-Phenyllactic acid	C9H10O3	166,0630	719	No ionization	165, 211, 201, 147, 119	End(100), 210(82)
Methyl 2-methoxybenzoate	C9H10O3	166,0630	829	167, 135	No ionization	206(100), 236(24), 296(18)
Methyl 4-methoxybenzoate	C9H10O3	166,0630	879	208, 167, 135, 194	No ionization	End(100), 210sh, 258(82)
6-Carboxypicolinic acid	C7H5NO4	167,0219	680	168, 388**	386**, 122	End(100), 220sh, 272(40)
3-Hydroxymandelic acid	C8H8O4	168,0423	675	No ionization	167, 133	End(100), 216sh, 278(6)
4-Hydroxymandelic acid	C8H8O4	168,0423	673	192, 151, 164	167, 213, 121	End(100), 230(20), 276(4)
2,5-Dihydroxyphenylacetic acid	C8H8O4	168,0423	680	210, 186, 232, 164 (PO)	167, 123, 213	End(100), 234(18), 294(12)
Orsellinic acid	C8H8O4	168,0423	717	210, 169, 151, 192	167, 123	212(100), 260(44), 296(17)
Gallic acid	C7H6O5	170,0215	676	No ionization	169, 125	212(100), 272(60)
Penicillic acid	C8H10O4	170,0579	702	171, 212, 188, 153	169, 215	End(48), 228(100)
Shikimic acid	C7H10O5	174,0528	672	157, 198 (VPO)	173, 219, 209, 155, 137, 111	210
Indolacetic acid	C10H9NO2	175,0633	767	176, 171, 146, 130	174, 220, 130, 144, 242	220(100), 278(19), 286sh
L-Ascorbic Acid	C6H8O6	176,0321	673	218, 177, 194,	175, 135	244
Fusaric acid	C10H13NO2	179,0946	707	180, 152, 175, 193	No ionization	End(100), 228(32), 272(45)
4-Hydroxyphenylpyruvic acid	C9H8O4	180,0423	705	No ionization	179, 195, 225, 201	236(30), 304(100)
Caffeic acid	C9H8O4	180,0423	691	222, 163, 181	179, 135	216(78), 240(61), 302sh, 324(100)
Stipitatic acid	C8H6O5	182,0215	681	224, 183 (PO)	181, 227, 249,	266(100), 310sh, 360(20), 415sh
3-Methylorsellinic acid	C9H10O4	182,0579	777	224, 183, 165	181, 137	220(100), 268(67), 304(18)

5-methyl-orselenic acid	C9H10O4	182,0579	767	224, 183	181, 137	216(100), 264(44), 306(13)
Carolic acid	C9H10O4	182,0579	676	183, 200, 224, 165, 155, 137	199	232(85), 264(100)
2,3-dimethoxy-5-methyl-1,4-benzoquinone	C9H10O4	182,0579	759	183, 140, 155, 168	No ionization	End(82), 268(100), 410(6)
Ethisolide	C9H10O4	182,0579	728	200 (VPO)	227 (VPO)	206
Methyl 3-methoxysalicylate	C9H10O4	182,0579	806	183, 246, 151, 210,	No ionization	215(100), 250(25), 316(12)
Barnol	C10H14O3	182,0943	812	183, 165, 224, 181, (PO)	227, 181, 166	204(100), 222sh, 272(4)
Valine-tenuazonic acid	C9H13NO3	183,0895	792	225, 185	192, 125	222(52), 278(100)
Spinulosin	C8H8O5	184,0372	699	No ionization	183, 168, 140	200(81), 212(60), 296(100)
Aspyrone	C9H12O4	184,0736	690	185, 167, 139, 226 (PO)	229, 219	204(100), 234sh
Asperlactone	C9H12O4	184,0736	685	226, 185, 202, 141	No ionization	218
Dihydroramulosin	C10H16O3	184,1099	743	185, 167, 208	No ionization	Not detected
2-Acetyl-3H-quinazolin-4-one	C10H8N2O2	188,0586	756	189, 147	187	206(100), 232(72), 304(38)
Aspinonene	C9H16O4	188,1049	676	206, 252, 153, 127	233, 169	Not detected
Kynurenic acid	C10H7NO3	189,0426	688	190, 162, 203,	188, 144	218(91), 242(100), 334(28)
Chrysogine	C10H10N2O2	190,0742	686	191, 173	189, 145, 173	End(85), 228(100), 264(22), 304(15)
3,5-Dimethyl-6-methoxyphthalide	C11H12O3	192,0786	889	193, 210, 234, 178, 163, 149	No ionization	210(100), 244(22), 300(15)
Scytalone	C10H10O4	194,0579	897	195, 236, 177, 218	193, 175, 151, 165	216(98), 230sh(78), 284(100), 316sh(45)
4-Hydroxymellein	C10H10O4	194,0579	752	195, 177, 149, 236 (PO)	239, 229, 193, 149, 121	208(100), 244(21), 312(16)
Dimethylphthalate	C10H10O4	194,0579	843	163, 195, 135	No ionization	End(100), 228(22), 276(5)
Caffeine; Coffein	C8H10N4O2	194,0804	684	195, 236	No ionization	208(100), 230sh, 272(65)
Novae-zelandin B	C11H14O3	194,0943	878	195, 236, 258	No ionization	208(100), 288(21)
Galiellalactone	C11H14O3	194,0943	756	195, 177	275, 239, 229, (PO)	222
4,8-Dihydroxy-3-methyl-3,4,4a,5-tetrahydro-1H-2-benzopyran-1-one	C10H12O4	196,0736	745	197, 238, 260, 180	195, 241, 231	212(98), 226sh, 332(100)
L-Tenuazonic acid	C10H15NO3	197,1052	826	239, 198, 144	196, 139	End(69), 222(60), 284(100)
Tenuazonic acid	C10H15NO3	197,1052	818	*239, 198	196, 139, 264	End(69), 222(60), 284(100)
Naphthalic anhydride	C12H6O3	198,0317	1428	370, 329 (PO)	327, 395, 667, 181, 249	203(100), 218(94), 258(92), 298sh, 360(62), 378sh
3,4,4a,5,6,7-Hexahydro-4,8-dihydroxy-3-methyl-1H-2-benzopyran-1-one	C10H14O4	198,0892	751	240, 199, 181, 182, 141	243, 233, 197 (PO)	268
Carolic acid (R)	C9H12O5	200,0685	676	183, 200, 224, 165, 155, 137	199	232(85), 264(100)
Tryptophan	C11H12N2O2	204,0899	678	205, 246, 188	203, 249, 239, 271, 261, 225	220(100), 280(15), 287sh
Xanthurenic acid	C10H7NO4	205,0375	684	206, 178, 219, 173	204, 160	220(63), 248(100), 344(21)
5-Formylmellein	C11H10O4	206,0579	846	No ionization	205	240(100), 278(50), 314sh
2-Pyruvoylaminobenzamide	C10H10N2O3	206,0691	746	207, 190, 224	205, 187, 411	218(100), 245sh(28), 296(7)
3-Methyl-5,7-dimethoxypthalide	C11H12O4	208,0736	794	209, 272, 191, 163	No ionization	218(100), 260(49), 292(16)

Isochromantoxin	C12H16O3	208,1099	872	209, 207, 271, 191, 179, 151	207, 191, 163	204(100), 226sh, 284(12)
2,4-Dihydroxy-6-acetonyl-benzoic acid	C10H10O5	210,0528	708	211, 193, 252	209, 123, 165	214(100), 262(50), 298(22)
Canadensolide	C11H14O4	210,0892	890	211, PO	209, 255, 231, 277, 121, 137	204
Terrestric acid	C11H14O4	210,0892	739	211, 200, 193, 159, 252	227*****	208(52), 231(22), 274(100)
Jasmonic acid	C12H18O3	210,1256	853	211, 252 (PO)	209, 255, 419	End
Tropodithietic acid	C8H4O3S2	211,9602	774	213, 236, 195, 167, 151	211, 167, 139, 107	210(100), 244(62), 306(98), 314sh, 358(39), 450(11)
Asperline	C10H12O5	212,0679	722	213, 230, 194, 153, 135	No ionization	206
Gallic acid propyl ester	C10H12O5	212,0685	765	254, 213, 212, 153, 168, 171	211, 124, 169	216(100), 274(68)
9,10-Dihydrojasmonic acid	C12H20O3	212,1412	908	254, 213, 194	211, 257, 279, 167	End(100), 210sh(10), 288(19)
Oxo C6 homoserine lactone	C10H15NO4	213,1001	686	214, 231, 259, 277, 236,	212	Not detected
Agistatin B	C11H18O4	214,1205	715	*197, 179	259, 249 PO	Not detected
Hydroxy C6 homoserine lactone	C10H17NO4	215,1158	680	216, 233, 238, 198, 279, 254	214	Not detected
Decarestrictine D	C10H16O5	216,0998	678	217, 181	261 (VPO)	Not detected
Gladiolic acid	C11H10O5	222,0528	764	No ionization	221, 289, 267, 177, 147, 119	268(100), 306 (43)
Nigragillin	C13H22N2O	222,1732	673	223, 264, 286, 183, 166, 129	No ionization	270
Farnesol	C15H26O	222,1984	1327	*205, 149	No ionization	End
Pyranonigrin A	C10H9NO5	223,0481	697	224, 241	222	208(100), 262(65), 310(75)
Cerulenin	C12H17NO3	223,1208	794	265, 224, 206, 196	222, 268, 204, 179	End(100), 208sh
Puberulonic acid	C9H4O7	223,9957	782	225, 242	223, 241	End(60), 276(100), 318(49), 363sh, 376(32), 412(30)
Novae-zelandin A	C11H12O5	224,0685	716	225, 266	223	206(100), 286(28)
Aspergillic acid	C12H20N2O2	224,1525	905	266, 225, 193, 166, 288, 197	223, 291, 269, 307, 205, 191, 151	228(80), 324(100), HCl: (242, 7300) (350/2, 9700)
Prephenic acid	C10H10O6	226,0477	681	290, 244 (PO)	181, 163, 225, 227	End(100), 224sh
2,4-Dihydroxy-6-(1-hydroxyacetonyl) benzoic acid	C10H10O6	226,0477	687	227, 268, 209, 250, 193, 163	225, 181, 137, 207	214(100), 260(49), 294(21)
Papyracillic acid	C11H14O5	226,0841	730	227, 209	225, 271, 261, 166, 181, 193, 123	230
Terrestric acid	C11H14O5	226,0841	739	211, 200, 193, 159, 252	*****227	208(52), 231(22), 274(100)
Aurantioclavine	C15H18N2	226,1470	714	227, 210, 171, 154, 168, 182, 198	No ionization	End(88), 224(100), 288(22)
Dihydrojasmonic acid-methyl ester	C13H22O3	226,1569	1077	268, 227, 194	No ionization	End(100), 222(42), 292(20)
Carlosic acid	C10H12O6	228,0634	684	229, 246, 211, 292	227, 139, 183, 249	232(100), 264(94), 316sh, 408(8)
Hydroxyisocanadensic acid	C11H16O5	228,0998	782	270 (VPO)	227, 141, 121	230
Ergothioneine	C9H15N3O2S	229,0885	674	230, 186, 127	228, 274, 125, 169	260
Hydroxypestalotin	C11H18O5	230,1154	707	231, 253, 248, 294, 181, 195, 213, 167	275, 265, 203, 163, 161	242

Sorbicillin	C14H16O3	232,1099	1135	233, 215, 205, 165	231, 189, 187, 213, 176	204(100), 324(83), broad to 450
Isovelleral	C15H20O2	232,1463	1004	233, 215, 187, 205, 173, 274	281, 231, 263 (VPO)	End(100), 210sh, 252(90)
Ascochin	C12H10O5	234,0528	976	No ionization	233, 205	256(100), 289sh, 312(11)
Cyclo-L-phenylalanin-L-serin	C12H14N2O3	234,1004	683	235, 207, 276, 162, 143	233, 279, 203 (PO)	End
2',3'-Dihydrosorbicillin	C14H18O3	234,1256	1169	235, 165, 217,	233, 178, 163, 150, 213	216(100), 232sh, 284(80), 328(30)
Visoltricin	C13H18N2O2	234,1368	740	235	No ionization	End(100), 282(100)
1-hydroxyeremophil-7(11),9(10)-dien-8-one	C15H22O2	234,1620	979	235, 276, 217, 207, 189, 175, 163,	No ionization	244 (100), 288(40)
5-Methoxycarbonylmellein	C12H12O5	236,0685	951	237, 278, 205 (PO)	235	230 (100), 255(sh), 312(10)
Austdiol	C12H12O5	236,0685	688	237, 300, 259, 278, 177, 219, 191, 201, 163	235, 175, 174, 173, 147, 135, 161, 163, 133	204(55), 256(70), 380(100)
Radicinin	C12H12O5	236,0685	743	237, 219, 193, 300, 254, 163	325 (PO)	208(70), 220(71), 236sh, 272(26), 344(100)
Walleminol	C15H24O2	236,1776	910	*260, 219, 201, 145, 159	201 (VPO)	End
Viridicatin	C15H11NO2	237,0790	936	238, 208, 220, 192	236, 208	204(78), 222(100), 240sh(50), 288(20), 318(27)328sh
Cyclopaldic acid	C11H10O6	238,0477	822	239, 280, 221, (PO)	237, 193, 165, 149, 135,	End(35), 244(100), 274sh, 322(8)
Pachybasin	C15H10O3	238,0630	1202	VPO	238	End(94), 224(61), 248sh, 260(100), 277sh(47), 332(10), 404(20)
Agroclavine	C16H18N2	238,1470	707	239, 208, 198, 183	No ionization	228(100), 280(28)
Culmorin	C15H26O2	238,1933	958	*221, 203, 262, 177, 147, 256	*283	End
Alizarin	C14H8O4	240,0423	928	No ionization	239	228sh, 248(100), 280(68), 328(14), 432(26)
Chrysazin	C14H8O4	240,0423	1093	241, 282 (VPO)	239	224(100), 252(88), 284sh, 428broad(48)
6,7-seco-Agroclavine	C16H20N2	240,1626	746	241, 210, 167, 195, 154, 181	No ionization	End(100), 220(84), 282(20)
Pyroclavine	C16H20N2	240,1626	691	241, 210, 168, 154	No ionization	226(100), 282(25)
Costaclavin	C16H20N2	240,1626	699	341, 210	No ionization	226(100), 282(25)
Festuclavine	C16H20N2	240,1626	706	241, 210	No ionization	222(100), 280(20)
Oxo-C8-homoserine lactone	C12H19NO4	241,1314	792	242, 259, 264, 287, 280, 305, 182, 214, 200	240	Not detected
Iodinine	C12H8N2O4	244,0484	970	No ionization	No ionization	288(100), 344(8), 524(8)
Verruculotoxin	C15H20N2O	244,1576	681	245, 286, 217, 200	No ionization	End
wpi-10-Verruculotoxin	C15H20N2O	244,1576	677	245, 286, 216, 199, 133	PO	End(100), 210sh
Hirsutanol-C	C15H20O3	248,1412	760	249, 231, 272, 290, 203, 175, 213	293, 229	216(43), 288(100)
Diaporthin	C13H14O5	250,0841	864	251, 233,	249, 295, 317 (PO)	244(100), 276(13), 286sh, 328(13)

Citrinin	C13H14O5	250,0841	928	251, 314, 233, 274, 292	267, 249, 295, 205, 223, 177, 231	216(100), 242sh, 328(40)
Oxaspirodion	C13H14O5	250,0841	807	268, 251, 233 PO	231, 249, 177, 159, 307	End(100), 126sh, 248(80)
Vertinolide	C14H18O4	250,1205	841	201, 224, 165, 251, 273, 292	249, 499, 521	230 (40), 278(100)
Trichodermol	C15H22O3	250,1569	804	292, 251, 233, 215, 274, 187	No ionization	End
3-O-Methylviridicatin	C16H13NO2	251,0946	930	252, 315, 236 (CH4 loss), 237	250 (PO)	294(96), 226(100), 280(20), 315sh, 324(22), 334sh
Frequentin	C14H20O4	252,1362	923	253, 235, 338, 216	251, 319, 207, 233, 179, 193	232
Walleminone	C15H24O3	252,1725	900	316, 253, 235	No ionization	End
Viridicatol	C15H11NO3	253,0739	828	254, 208, 236	252, 224,	222(100), 240sh, 285(20), 305sh, 318(26), 329sh
Chrysophanic acid	C15H10O4	254,0579	1184	255, 296 (VPO)	253, 321, 225 (PO)	224(100), 256(65), 280sh, 288sh, 43broad2(30)
Phomarin	C15H10O4	254,0579	1048	255, 296	253, 225	220(90), 268(100), 294sh, 415(21)
Soranjidiol	C15H10O4	254,0579	1076	255, 296 (VPO)	253	220(90), 268(100), 293sh(50), 412(26)
w-Hydroxypachybasin	C15H10O4	254,0579	928	255, 296 (PO)	253, 224	End(85), 222(61), 258(100), 277sh(45), 334(10), 440(20)
Elymoclavine	C16H18N2O	254,1419	676	255, 224, 212, 296, 237	No ionization	End(94), 220(100), 280(21)
Epoxyagroclavine	C16H18N2O	254,1419	710	255	No ioniztion	224(100), 280(20)
Lysergol	C16H18N2O	254,1419	675	255	No ionization	End(100), 230(86), 242(84), 314(32)
Palitantin	C14H22O4	254,1518	855	255, 237, 272, 219, 296,	VPO	232
ochratoxin α	C11H9ClO5	256,0139	830	257, 239	255, 211, 167	216(100), 235sh, 248sh, 336(22)
Lambertellin	C14H8O5	256,0372	943	279, 257, 298 (VPO)	255, 287 (VPO)	212(100), 288(79), 432(21)
Purpurin	C14H8O5	256,0372	10	257, 298 (PO)	255	204(72), 256(100), 296(30), 456sh, 480(28), 508sh
Viridicatic acid	C12H16O6	256,0947	776	257, 239 (PO)	255	233(88), 244(52), 265(100)
Chanoclavine-I	C16H20N2O	256,1576	676	257, 208, 168, 193, 156, 226, 198	No ionization	End(87), 224(100), 280(22)
Dihydrolysergol I	C16H20N2O	256,1576	675	257	No ionization	224(100), 280(20)
Fumigaclavine B	C16H20N2O	256,1576	673	257	No ionization	224(100), 280(19)
Iso-dihydrolysergol	C16H20N2O	256,1576	676	259	301, 269	224(100), 282(20)
Roquefortine B	C16H20N2O	256,1576	672	257, 298, 239, 197	No ionization	224(100), 280(19)
Indolmycin	C14H15N3O2	257,1164	799	258, 299, 184, 156, 144	302, 256, 292, 228, 199	220(100), 282(9)
nor-Rubrofusarin	C14H10O5	258,0528	934	259	257, 325, 303	226(57), 280(100), 328(4), 412(13)
Ravenelin	C14H10O5	258,0528	1059	257 (VPO) strange	257, 303, 213 (PO)	End(75), 236sh(60), 260(100), 340(37), 398(10)
Alternariol	C14H10O5	258,0528	869	259, 300 (PO)	257, 303, 325	204(50), 256(100), 288(20), 300(20), 340(23)
Norlichexanthone	C14H10O5	258,0528	936	259	257	204(65), 242(100), 267sh, 312(60), 346sh

Bredinine	C9H13N3O6	259,0804	671	260.169	258, 216	End(100) broad to 250, 280 (10)
2-hydroxy-1-(8-hydroxy-3-methylisoquinolin-7-yl)-3-methylbutan-1-one	C15H17NO3	259,1208	734	260, 242, 214, 204, 186	258, 240, 215	220 (95), 248(100), 262 (98), 364 (49)
Maculosin	C14H16N2O3	260,1161	683	302, 261, 196, 177, 136	No ionization	End(100), 220sh(30), 275(5)
Cinoxacin	C12H10N2O5	262,0590	743	263, 235, 326	307, 297, 261 (VPO)	250(48), 264(100), 265(53)
2,7-dimethoxy-6-ethyljuglone	C14H14O5	262,0841	1086	263, 235, 220	No ionization	224(100), 260(50), 410(12)
4Z-Infectopyrone	C14H16O5	264,0998	829	265, 203, 247, 223, 139, 177	263, 299, 309, 331	220(100), 269(71), 346(80)
Infectopyrone	C14H16O5	264,0998	854	265, 203, 247, 139, 219, 191	309, 263, 204, 331, 143, 189 (PO)	218(100), 268(68), 328(74)
Abscisic acid	C15H20O4	264,1362	800	265, 282, 247, 288, 306, 328, 229, 201, 187, 173, 219,	263, 309, 331, 219, 153, 204, 201	264
Trichothecolone	C15H20O4	264,1362	712	306, 265, 247, 229, 211, 203, 187, 175	163, 161, 309, 299, 171	228
Thiamine	C12H17N4OS	265,1118	672	265, 174, 154, 144, 122	233, 147, 217, 251, 263, 281	End(100), 248(63), 273sh
Diaportinol	C13H14O6	266,0790	784	267, 249, 219	311, 265, 301	244(100), 276(12), 286sh, 328(12)
7α-Hydroxytrichodermol	C15H22O4	266,1518	696	308, 267, 249, 284, 231, 219	311 (VPO)	End
Verrucarol	C15H22O4	266,1518	700	267, 308, 249, 231, 213, 185, 330	236, 207, 311, 190 (PO)	End
Isoergine	C16H17N3O	267,1372	675	268	No ionization	End(95), 232(100), 312(312(38)
Ergine	C16H17N3O	267,1372	675	268, 284, 331, 225, 223, 208	No ionization	End(100), 230(86), 242(84), 314(32)
Pachybasic acid	C15H8O5	268,0372	975	No ionization	267, 241, 223, 213, 195, 167	212(80), 228(78), 260(100), 340(10), 404(22)
Lysergic acid	C16H16N2O2	268,1212	675	269	313, 267, 233 (PO)	226(100), 240sh, 310(38)
Rugulovasine B	C16H16N2O2	268,1212	683	269, 251, 310, 291, 332, 328, 223, 210	313, 303, 267, 223, 179, 193	220(100), 292(15)
DihydroErgine	C16H19N3O	269,1528	675	271, 311	No ionization	224(100), 282(22)
Oxo-C10-homoserine lactone	C14H23NO4	269,1627	930	287, 270, 292, 333, 308, 210	268	Not detected
Islandicin	C15H10O5	270,0528	1319	No ionization	269 (PO)	230(100), 252(69), 292(23), 264sh, 492(35)
Iso-emodin	C15H10O5	270,0528	941	No ionization	269, 337 (VPO)	End(55), 224(100), 256(64), 286sh, 432(30)
Emodin	C15H10O5	270,0528	1087	271 (VPO), 312	269	222(100), 258sh, 268(55), 288(64), 440(38)
Helminthosporin	C15H10O5	270,0528	1276	No ionization	369, 337, 349, (PO)	231(100), 255(49), 288(23), 484(30), 518sh
Dihydrolysergic Acid	C16H18N2O2	270,1368	675	271	No ionization	224(100), 280(24)
Hydroxy-C10-homoserine lactone	C14H25NO4	271,1784	895	272, 289, 294, 335, 310, 254	270	Not detected
Alternariol monomethylether	C15H12O5	272,0685	1024	273, 314 (poor ionzation)	271, 256	204(50), 256(100), 288(20), 300(20), 340(23)

Rubrofusarin	C15H12O5	272,0685	1128	273	271	228(49), 278(100), 328(4), 408(13)
Phenicin	C14H10O6	274,0477	718	275, VPO	273, 229, 245, 214, 217, 201	214(100), 270(80)
3-acethoxyeremophil-1(2),7(11),9(10)-trien-8-one	C17H22O3	274,1569	1095	275, 316, 233, 215, 187, 173, 145	No ionization	280(100), 306sh(65)
2,2-Dimethyl-5-amino-6-(2'E-ene-4'-hydroxylbutyryl)-4-chromone	C15H17NO4	275,1158	872	276, 220, 238, 258, 202, 192, 174	No ionization	214(89), 252(100), 282(42), 390(57)
Ascochitine	C15H16O5	276,0998	1070	277, 259	275, 293, 321, 343, 231, 201, 202	220(81), 262sh, 280(100), 326(15), 242(15), 416(38)
3-acethoxyeremophil-7(11),9(10)-dien-8-one	C17H24O3	276,1725	1076	277, 318, 217, 189, 161, 147	No ionization	248 (100), 280(60)
Sclerotigenin	C16H11N3O2	277,0851	770	278, 319, 249, 233, 341, 382	No ionization	215sh(78), 230(100), 267(22), 280(20), 310(10), 319sh
2,7-dimethoxy-5-hydroxy-6-(l-hydroxyethyl)-1,4-naphthoquinone	C14H14O6	278,0790	902	261, 233, 324, 302, 293, 356	No ionization	224(100), 260(50), 410(12)
Citreo isocumarin	C14H14O6	278,0790	761	279, 296, 301, 219, 261	277, 323, 219	244(100), 258sh, 275(15), 286sh, 328(13)
Pentoxifylline	C13H18N4O3	278,1379	734	279, 301, 320, 317, 342, 222, 181, 156, 138	*293, 323	214(100), 276(72)
Diaportinic acid	C13H12O7	280,0583	800	281, 298, 235, 276	279, 347, 337, 325, 315, 261, 173, 217, 205	244(100), 276(13), 286sh, 328(13)
Cyclopeptine	C17H16N2O2	280,1212	854	281, 322, 561. 189, 134	279, 183, 261, 149, 175, 222	216(100), 292(5)
7-epi-Brefeldin A	C16H24O4	280,1675	856	322, 281, 245, 263, 199	325, 315 (PO)	End, broad to 270 nm
Cyanein	C16H24O4	280,1675	857	322, 281, 263, 245, 199, 163	No ionization	End, broad to 270 nm
Linoleic acid	C18H32O2	280,2402	1597	322, 281, 263, 173,	325, 279, 249, 181	End
Scirpentriol	C15H22O5	282,1467	683	306, 283, 265, 247, 217	327, 317 (PO)	End
Fumagillol	C16H26O4	282,1831	875	283, 251, 265, 233, 215	No ionization	End
Oleic acid	C18H34O2	282,2559	1733	324, 265, 247, 298	327, 281, 317	End
C12-homoserine lactone	C16H29NO3	283,2147	1213	284, 301, 306, 143, 102	328, 318	Not detected
Macrosporin	C16H12O5	284,0685	1040	285	283, 351, 268, 240	224(53), 284(100), 309sh, 280(20)
Physcion	C16H12O5	284,0685	1286	285, 326	283, 269, 240	End(60), 224(100), 257sh, 268(54),288(55), 436(36)
Questin	C16H12O5	284,0685	975	285	283, 240	224(100), 250sh, 284(64), 436(27)
Asperic acid	C16H28O4	284,1982	985	253, 267, 307, 235, 285	383	222
Bostrycoidin	C15H11NO5	285,0637	989	286, 327, 243	284, 352, 269, 241 (PO)	204(96), 252(100), 324(21), 472sh, 492(29), 526sh
Piperine	C17H19NO3	285,1365	1001	286, 349, 201	284, 330	250(30), 312(67), 344(100)

Catenarin	C15H10O6	286,0477	1132	No ionization	285, 257 (PO)	232(100), 256(50), 276(52), 302(32), 464sh, 492(42), 520sh
Cynodontin	C15H10O6	286,0477	1349	No ionization	285, 353, 257	236(100), 284sh, 296(18), 488sh, 516(40), 544(38), 552(39)
Flavasperone	C16H14O5	286,0841	855	287 (PO)	285	End(100), 224(71), 276(67), 325(5), 404(8)
Lichexanthone	C16H14O5	286,0841	1333	287, 272, 244, 328	285, 380 (VPO)	208(71), 242(100), 308(65), 341sh
Salicin	C13H18O7	286,1053	678	304, 309	331, 321	End(100), 211(sh), 268(15)
Fusoxysporone	C20H30O	286,2297	1582	287, 328, 269, 175, 203	No ionization	End(100), 244(85)
Pyrophen	C16H17NO4	287,1158	816	288, 229, 310, 351,	286	200(100), 270(25), 323(20)
Anhydrofusarubin	C15H12O6	288,0634	1125	289 (PO)	287, 272, 244, 355, 333 (PO)	End(100), 236(88), 288(87),348sh, 540(47)
Violaceic acid	C15H12O6	288,0634	798	330, 289, 271	287, 309, 255, 243, 137	End(100), 228(82), 262(68), 284sh
Funalenone	C15H12O6	288,0634	854	289	287	214(100), 235(sh), 265(35), 280(sh), 368(sh), 402(40)
Violaceic acid	C15H12O6	288,0634	739	No ionization	287, 243	208(100), , 274, 350
Xanthocillin-X	C18H12N2O2	288,0899	1023	289, 348, 371(VPO)	287, 260, 233	End(50), 240(20), 360(100), 380sh
Citromycetin	C14H10O7	290,0427	897	291, 273, 313, 354	289, 271,217, 175	212(100), 256(53), 300(35), 368(55)
Altenusin	C15H14O6	290,0790	838	291,273, 245, 239, 198, 227, 296	289, 245, 230, 271	End(100), 217sh, 258(31), 290(20)
Javanicin	C15H14O6	290,0790	932	291, 249, 273	289, 357, 247, 232, 274	228(100), 304(31), 480sh, 504(28), 538sh
Altenuene	C15H16O6	292,0947	790	293, 275, 257, 229, 201	337, 327, 291,	240(100), 280(36), 320(18)
6-Methoxy-citreoisocoumarin	C15H16O6	292,0947	848	310, 293, 315, 331, 233, 275, 207	291, 337	244(100), 258sh, 278(13), 288sh, 328(14)
Italicic acid	C15H16O6	292,0947	889	310, 293, 334, 356, 275, 247, 223, 205	291, 337, 247, 219, 175	240(52), 268(48), 336(100)
Solaniol	C15H16O6	292,0947	900	275, 293, 338, 257 (PO)	291, 247, 232, 273, 204, 346, 359 (PO)	End(60), 228(100), 308(30), 480sh, 504(28), 542sh
Curvularin	C16H20O5	292,1311	891	293, 275, 310, 334, 210, 169	291, 247, 190, 203,	End(100), 222(72), 272(41), 303(30)
Cladosporin	C16H20O5	292,1311	850	293, 275, 257, 231 (PO)	291, 337	216(100), 268(65), 300(23)
Fusarochromanone	C15H20N2O4	292,1423	687	293, 275, 258, 234, 315, 356	No ionization	212(80), 252(100), 280(41), 388(56)
Trichodermin	C17H24O4	292,1675	968	293, 233, 215, 274 (233 w CH3CN), 184, 187, 197, 203	No ionization	End
Cyclopenin	C17H14N2O3	294,1004	830	295, 336, 358, 264, 251, 236, 205	293, 208, 235, 175	End(95), 212(100), 290(6)
Dihydro-italicic acid	C15H18O6	294,1103	1124	No ionization	293, 361, 249	End(60), 278(100), 330(46)
Deoxynivalenol	C15H20O6	296,1260	681	297, 249, 360, 279, 231, 203 (PO)	341, 331, 357	220

Oxo-C12-homoserine lactone	C16H27NO4	297,1940	1079	298, 315, 320, 336, 298, 361, 238	296, 169, 195	End
Hydroheptelidic acid	C15H22O6	298,1416	740	316, 299, 281, 262, 263, 245, 221	595, 297, 279, 343, 333, 617	216
T-2 tetraol	C15H22O6	298,1416	675	321, 316, 281, 263, 299 PO	No ionization	End
Fumigaclavine A	C18H22N2O2	298,1681	690	299, 340	No ionization	224(100), 280(18)
Roquefortine A	C18H22N2O2	298,1681	700	299, 340, 239	297	224(100), 280(18)
Leucyltryptophanyl-diketopiperazine	C17H21N3O2	299,1634	778	300, 341, 130	298, 344, 334, 330, 169	220(100), 282(20), 294sh
Hydroxy-C12-homoserine lactone	C16H29NO4	299,2097	1026	300, 317, 322, 363, 338, 282	298, 334, 344	End
2,8-Dihydroxy-3-methyl-9-oxo-xanthene-1-carboxylic acid methyl ester	C16H12O6	300,0634	990	301, 269	299, 345, 367, 267 (PO)	End(100), 236(90), 262(96), 292(36)
Dermoglaucin	C16H12O6	300,0634	1004	301 (VPO)	299, 285 (PO)	212(100), 284(99), 432(40)
Erythroglaucin	C16H12O6	300,0634	1311	No ionization	299, 285 (VPO)	230(100), 256(52), 276(53), 304(41), 370sh, 492(42), 518sh
Roseopurpurin	C16H12O6	300,0634	807	301	299, 256	220(100), 252(73), 288(82), 436(39)
3,4,5-Trihydroxy-7-methoxy-2-methylanthraquinone	C16H12O6	300,0634	1107	301 (VPO)	299, 367	222(10), 270(46)
Questinol	C16H12O6	300,0634	818	301	299, 256	224(100), 28850), 440(22)
Isodihydroauroglaucin	C19H24O3	300,1725	1383	301, 283, 231,	299, 349, 218, 217, 288	230(100), 276(18), 388(14)
Aflatoxin B3	C16H14O6	302,0790	827	303, 344, 285, 257, 245, 229	301 (VPO)	206(100), 260(23), 328(28)
Aurantiamine	C16H22N4O2	302,1743	788	303, 344, 235, 176	301	232(53), 320(100)
Aurantiamine	C16H22N4O2	302,1743	774	303, 344	301, 347	226(100), 316(88)
Viridamine	C16H22N4O2	302,1743	749	303, 260, 247, 176, 275	301, 258, 333	End(80), 222sh, 302(100), 315sh
Aspergin	C19H26O3	302,1882	1521	303, 247	301, 347, 283, 273,	230(232), 276(34), 396(24)
Dehydroflavoglaucin	C19H26O3	302,1882	1455	303. 285, 247	301, 273, 204, 281	End(100), 234(21), 278(24), 388(18)
Nectriafurone	C15H12O7	304,0583	902	305, 287,	303, 371, 288	End(100), 240(81), 258(80), 324(25), 356sh, 444(57), 464sh
Griseophenone C	C16H16O6	304,0947	893	305, 165, 185, 208	303, 349, 271, 265, 165	205(100), 227sh(44), 297(50), 337sh(12)
Flavoglaucin	C19H28O3	304,2038	1538	305, 249	349, 303, 275, 204, 163	End(100), 240(36), 276(36), 388(16)
Oosporein	C14H10O8	306,0376	720	No ionization	305, 249, 221, 205, 277, 233, 327	End(92), 288(100)
Canescin A	C15H14O7	306,0740	865	307, 275, 229	305, 273, 261, 229, 217	248(100), 280(12), 320(11)
Fusarubin	C15H14O7	306,0740	856	289, 307, 247, 330, 370	305, 373, 291, 248, 233, 262, 275	228(100), 304(30), 474sh, 500(28), 526sh

Chromanol 1	C16H18O6	306,1103	898	289, 307, 271, 324	305, 351, 341, 163, 193, 135, 231, 246, 261, 237	222(10), 270(46)
Chromanol 2	C16H18O6	306,1103	877	289, 307, 271, 370, 256, 343, 215	305, 351, 341, 193, 261, 163, 135,193, 231, 246	222(10), 270(46)
Methyl italicate	C16H18O6	306,1103	978	307, 324, 275, 247, 329, 370	323, 307, 305, 339, 351	240(50), 272(45), 336(100)
Expansolide A	C17H22O5	306,1467	958	307, 306, 247, 265	323, 391, 263, 201	End
Circumdatin C	C17H13N3O3	307,0957	795	308, 349, 271, 265, 291	306	232(100), 272(30), 302(13), 310(12), 322SH
6,7-Dihydroxy-3-(1'-hydroxy-3'-butanoyl)chromone-5-carboxylic acid	C14H12O8	308,0532	729	No ionization	No ionization	219(100), 253sh, 256(100). 352(30), 381(25)
Dihydrofusarubin A	C15H16O7	308,0896	791	279, 309, 291, 320, 262, 249, 221	307, 375, 277, 262, 205, 219	208(65), 244(100), 276(36), 300(24), 388(44)
Dihydrofusarubin B	C15H16O7	308,0896	766	279, 291, 309, 262, 221, 249	307, 375, 277, 262, 205, 219	208(65), 244(100), 276(36), 300(24), 388(44)
5'-Hydroxyasperentin	C16H20O6	308,1260	785	326, 309, 331, 291, 273, 229, 173, 247, 255	307, 353, 343, 189, 263, 289	214(100), 267(68), 300(28)
Cyclopenol	C17H14N2O4	310,0954	750	311, 177, 352, 146, 205, 252, 280	309, 175, 200, 224, 238, 252, 266, 355	End(100), 214sh, 284(7)
Mollicin	C14H10Cl2O4	311,9956	1079	No ionization	311, 291, 275, 240, 228, 211	208(100),262(80), 255sh, 420(18)
Aflatoxin B1	C17H12O6	312,0634	847	313, 376, 285, 335	311 (PO), 285,	200, 224(70), 264(45),336sh, 364(68)
Nivalenol	C15H20O7	312,1209	673	295, 336, 313, 247, 265, 330, 189, 175, 201 (PO)	357, 347, 313, 281	220
2-Amino-14,16-dimethyloctadecan-3-ol	C20H43NO	313,3345	1089	355, 314, 296, 337	No ionization	End
Aflatoxicol A	C17H14O6	314,0790	864	297 (M+H-H2O), 378, 269, 241	313, 347, 331 (VPO)	208, 252shc, 260(30), 332(32)
Aflatoxin B2	C17H14O6	314,0790	823	315, 378, 317	313, 298, 285, 270, 257, 242 (VPO)	200, 222(68), 232sh,268(48),336sh, 364(90)
Heptelidic acid chlorohydrin	C15H21ClO5	316,1078	879	317, 334, 299, 253, 217, 281	315, 631, 653	220(100), 266(30), 304(13)
Rosenonolactone	C20H28O3	316,2038	1167	358, 317, 299, 271, 380	215, 333 (PO)	End
Dichlorodiaportin	C13H12Cl2O5	318,0062	1105	319, 360, 177, 246	363, 317, 245, 205	244(100), 276(12), 286sh, 328(12)
Asperthecin	C15H10O8	318,0376	846	No ionization	317, 299	End(75), 236(88), 260(100), 286(55), 316(36), 484(56), 506sh
Antibiotic Y	C15H10O8	318,0376	907	319, 382, 305, 287	317, 385, 407, 285, 258 (PO)	204(78), 216(78), 244(100), 280(95), 348(75), 364(82)
Dechlorogriseofulvin	C17H18O6	318,1103	884	319, 360, 341, 382, 165, 181, 251	No ionization	212(90), 256(60), 288(100), 325sh
Zearalenone	C18H22O5	318,1467	1020	319, 301, 283, 231, 187	317, 273, 175, 187, 289, 299, 149	236(100), 272(45), 318(18)

3,4-Epoxy-6-hydroxy-dolabella-7E,12-dien-14-one	C20H30O3	318,2195	876	301, 319, 283, 360, 255	No ionization	End(100), 242(70)
PR-imine	C17H21NO5	319,1420	745	320, 278, 260, 232, 218, 192	318, 364, 354, 276, 258, 216 (PO)	252(100), 287sh(38)
1-Deoxybostrycin	C16H16O7	320,0896	794	321, 303, 344, 285, 259	319, 301, 387, 286, 243, 258, 271	228(100), 304(28), 474sh, 500(30), 536(18)
2,7-dimethoxy-5-hydroxy-6-(l-acetoxyethyl)-1,4-naphthoquinone	C16H16O7	320,0896	946	*261, 279, 302, 324, 384	No ionization	222(100), 254(49), 314(31), 426(13)
Mycophenolic acid	C17H20O6	320,1260	932	321, 343, 303, 275, 248, 207	319, 341, 275, 207, 191	216(100), 252(24), 304(12)
PR-toxin	C17H20O6	320,1260	931	321, 279, 261	319, 291, 277, 259, 231 (PO)	256
α-Zearalenol	C18H24O5	320,1624	951	321, 303, 285, 267, 257, 229, 217	319, 275, 301, 291, 257, 233, 174	236(100),276(45), 316(18)
β-Zearalenol	C18H24O5	320,1624	906	321, 303, 285, 267, 217	319, 365	242(100), 270(50), 312(19)
Zearalanone	C18H24O5	320,1624	1011	321, 303	319, 275, 205, 301, 161	218(100), 264(56), 302(23)
Neoechinulin B	C19H19N3O2	321,1477	909	322, 363, 254, 295, 266	320	End(100), 228(100), 272(78), 282sh, 372 (47)
Chromanol 3	C17H22O6	322,1416	793	249, 231, 342, 325, 307, 388	323, 369, 359, 279, 249, 163, 193, 188, 221	222(10), 270(46)
α-Zearalanol	C18H26O5	322,1780	937	323, 305, 287	321, 361	218(100), 264(56), 302(23)
β-Zearalanol	C18H26O5	322,1780	895	323, 305, 287, 277	321, 367, 277, 303	218(100), 264(56), 302(23)
Neoechinulin A	C19H21N3O2	323,1634	871	324, 365, 256, 297, 268	322	226(100), 288(28), 328(33)
Sterigmatocystin	C18H12O6	324,0634	1053	325, 310, 388	No ionization	204(75), 235sh, 248(100), 328(47)
Formyl-xanthocillin X	C18H16N2O4	324,1110	728	325, 282, 342, 347, 370, 388 (PO)	No ionization	End(70), 220(30, 278sh), 338(100)
Ergometrine	C19H23N3O2	325,1790	675	326	324	End(100), 228(94), 314(36)
Ergonovinine	C19H23N3O2	325,1790	675	326	370, 324 (PO)	End(100), 234(100), 314(34)
Preechinulin	C19H23N3O2	325,1790	837	326, 356, 370, 389, 221, 198, 258, 270,	370, 360, 324, 127 (PO)	End(79), 224(100), 284(20)
Gliotoxin	C13H14N2O4S2	326,0395	796	327, 263, 245, 227, 286, 217	325, 261, 243, 217	End(100), 270(39)
Dihydrosterigmatocystin	C18H14O6	326,0790	1009	327, 390, 312, 299, 271	393, 325 (VPO)	206(55), 233sh,248(100), 326(44)
Trisdechloronornidulin	C19H18O5	326,1154	1018	327, 368	325, 371, 281, 269	204(100), 254sh(45)
Aflatoxin G1	C17H12O7	328,0583	803	329, 392, 351	327 (VPO)	200, 220(90), 232sh,264(40),336sh, 368(70)
Aflatoxin M1	C17H12O7	328,0583	779	329, 392, 367, 351, 346, 301, 273	327	204(100), 228(90), 264(52), 330sh, 360(81)
Aflatoxin G2	C17H14O7	330,0740	788	331, 394, 353,	329 (VPO)	200, 216(95), 244sh, 264(40), 372(85)
Aflatoxin M2	C17H14O7	330,0740	746	331, 348	329 , 315	End(98), 222(83), 265(53), 360(100)

Auranthine	C19H14N4O2	330,1117	820	372, 331, 348, 249	329, 276 (PO)	228(100), 268(23), 278sh, 312(10), 322sh
Sulochrin	C17H16O7	332,0896	882	333, 209, 301, 396, 237, 227, 151	331, 299, 377, 399, 181, 19, 167	208(100), 216(85), 284(40), 320sh
Byssochlamic acid	C18H20O6	332,1260	1155	No ionization	331, 287, 259, 215, 207, 243, 187 (PO)	204(100), 352(58)
Trichothecin	C19H24O5	332,1624	1166	333, 374, 247, 229, 211	No ionization	216
Rugulosuvine	C20H19N3O2	333,1477	819	334, 364, 375,	378, 332, 362, 408	224(100), 280(18)
Penicillin G	C16H18N2O4S	334,0987	845	335, 352, 398, 160, 176, 217, 239, 307	333, 379, 401, 192, 289	End(100), 206sh(75)
Mitomycin C	C15H18N4O5	334,1277	675	335, 242, 274, 357, 398	333	216(100), 242sh, 360(100)
Altersolanol A	C16H16O8	336,0845	703	337, 319, 301, 273, 259, 245	335	220(100), 268(41), 430(12)
Bostrycin	C16H16O8	336,0845	714	337, 400, 319, 301, 273, 259	335 (VPO)	228(100), 304(30), 482sh, 540(26), 536sh
Cyclopiazonic acid	C20H20N2O3	336,1474	1123	337, 182, 196, 400	335, 180, 140	224(100), 280(51)
Versicolorin A	C18H10O7	338,0427	1030	No ionization ****	No ionization****	222(100), 255(48), 290(84), 454(26)
O-Methylsterigmatocystin	C19H14O6	338,0790	934	339, 402, 324, 311, 306	355, 341, 369, 313, 327, 285, 271	204(85), 240(100), 312(50)
15-Acetyldeoxynivalenol	C17H22O7	338,1366	726	339, 321, 356, 279, 297, 402, 386, 231, 203, 261, 380 (PO)	383, 373	220
3-Acetyldeoxynivalenol	C17H22O7	338,1366	726	339, 321, 356, 279, 297, 402, 386, 231, 203, 261, 380 (PO)	383, 373	220
Aphidicolin	C20H34O4	338,2457	837	*362, 344, 380, 285, 303, 267	383 (PO)	End
Anacine	C18H22N4O3	342,1692	786	343, 326, 198, 365, 281, 406	341, 387, 270, 212, 228, 255	202(95), 228(100), 272(29), 308(12), 316sh
Violacein	C20H13N3O3	343,0957	893	No ionization	342 (PO)	212(100), 260(52), 374(19), 576(62)
Tensidol B	C18H17NO6	343,1056	889	344, 230, 361, 407, 326	342, 228	212(100), 248(sh)
Usnic acid	C18H16O7	344,0896	1345	345, 327, 303, 330, 261, 233 (PO)	343, 328, 709	232(100), 280(72), 260sh
Trypacidin	C18H16O7	344,0896	934	345, 301, 313, 363, 408	345, 391, 359, 181, 313, 285	208(100), 288(55), 328sh
Gibberellic acid	C19H22O6	346,1416	724	329, 388, 370, 347 (PO)	345, 391, 257, 239, 221	End
Asterric acid	C17H16O8	348,0845	936	331, 349, 371, 299,287, 272	347, 393, 369, 303, 271, 256, 166, 149	212(100), 252(30), 316(16)
Glauconic acid	C18H20O7	348,1209	1030	349, 331	393, 303, 365, 347, 259	212(100), 232(88)
Clerocidin	C20H28O5	348,1937	972	390, 349, 331 (VPO)	411, 347, 439 (PO)	236
pre-Paraherquamide	C22H27N3O	349,2154	733	350, 322	394, 384, 358,	226(100), 282(20)
VM-55599	C22H27N3O	349,2154	727	350, 391, 322, 334	394, 384, 348, 332,	226(100), 282(18)
Penicillin V	C16H18N2O5S	350,0936	880	368, 351, 160, 414, 201, 255, 233, 323	208, 349, 305, 699, 721	End(100), 210s, 268(4), 276sh
1-Hydroxybyssochlamic acid	C17H18O8	350,1002	1164	368 (PO)	349, 305, 261, 217, 233	204(100), 252(58)
Cycloechinulin	C20H21N3O3	351,1583	826	352, 393, 296, 188	350, 396, 335	218(100), 235(94), 268(60), 304(72), 378(66)
Tryprostatin B	C21H25N3O2	351,1947	910	352, 296, 284, 198	350, 396 (PO)	End(100), 228(90), 282(22)

Griseofulvin	C17H17ClO6	352,0714	934	353, 394, 319, 285, 215, 165	397, 387 VPO	212(98), 237(88), 251sh, 292(100), 325(24)
Altertoxin I	C20H16O6	352,0947	856	317, 353 (VPO)	351, 297, 263, 315, 333 (PO)	216(74), 260(100), 284(42), 356(14)
Rubropunctamine	C21H23NO4	353,1627	986	354, 376, 310, 417	352, 337, 308, 294, 253	262(70), 306(100), 416(88), 524(96)
5-Methoxysterigmatocystin	C19H14O7	354,0740	1025	355, 418, 340	No ionization	204(90), 235sh, 248(100), 275sh (30), 330(48)
3-hydroxy terphenylin	C20H18O6	354,1103	837	355, 323, 305, 416	353, 323, 338, 399	210(100), 227sh, 276(46)
Viridiol	C20H18O6	354,1103	792	355, 306, 322, 309, 281	399, 353, 279	End(62), 253(100), 320(46)
Fusarenon X	C17H22O8	354,1315	693	355, 372, 418 (PO)	399	220
Rubropunctatine	C21H22O5	354,1467	1255	355, 377, 414, 311, 257, 293, 241, 213	353, 327	End(100), 220(58), 244sh, 292(28), 474(95)
Bis-dethio-bis(methylthio)-gliotoxin	C15H20N2O4S2	356,0864	821	309, 357, 243, 420, 215, 233, 243, 251	No ionization	End(100), 268(20)
Tensidol C	C19H19NO6	357,1212	1035	358, 230	356, 228	212(100), 248(sh)
Monascine	C21H26O5	358,1780	1249	359, 422, 261, 243, 215, 287	357, 329, 313	236(95), 296(8), 396(100)
Circumdatin-B	C20H17N3O4	363,1219	859	364, 405, 427	362, 408, 347, 319, 293	End(85), 248(100), 280(28), 340(12)
Austamide	C21H21N3O3	363,1583	840	364, 427, 308, 252, 198	408, 398, 362, 430	232(100), 264sh(45), 396(8)
Monorden	C18H17ClO6	364,0714	879	365, 347, 321, 303, 277, 382, 406	363, 275, 283, 319, 335,	204(100), 276(46); EtOH-HCl: (265, 17700)
Atpenin A5	C15H21Cl2NO5	365,0797	1156	366, 276, 348	364,328, 292, 277, 262, 313	End(100), 238(61), 272(42), 338(58)
Brevianamide B	C21H23N3O3	365,1739	822	366, 383, 429, 407, 731, 748, 383	410, 364, 400, 364, 296, 165	236(100), 258sh, 408(12)
Brevianamide A	C21H23N3O3	365,1739	822	366, 407, 338, 321, 348, 235, 310, 276	410, 364, 296 (PO)	232broad(100), 262sh, 408(13)
Candidusin C	C21H18O6	366,1103	1005	367, 351, 335, 304,	411, 397, 280, 365, 349	218(100), 242sh, 282(53), 296(50), 334(71)
Glauconic acid, open form	C18H22O8	366,1315	868	367, 349	365	232 (100)
4,15-Diacetoxyscirpenol	C19H26O7	366,1679	822	384, 430, 389, 307, 247, 229, 199, 211, 183, 171	No ionization	End
Ferulenol	C24H30O3	366,2195	1482	367, 389, 216, 243, 231, 257, 299, 311, 285, 408	365, 228	204(100), 274(28), 284(30), 310(28), 220sh
Fumigaclavine C	C23H30N2O2	366,2307	773	367	365, 214 (PO)	226(100), 280(19)
Ophiobolin G	C25H34O2	366,2559	1467	430, 367, 408, 384, 349, 307, 293, 201	No ionization	236
Brevicompanine B	C22H29N3O2	367,2260	1035	368, 300, 312, 341, 198, 130	412, 366 (PO)	208(100), 244(19), 300(8)
Averufin	C20H16O7	368,0896	1312	No ionization	367	224(95), 292(100), 268(55), 254sh, 322(24), 456(32)
Ochratoxin B	C20H19NO6	369,1212	944	370, 324, 233, 205, 268	368, 324, 390, 280, 220	216(100), 248sh, 320(20)
Norsolorinic acid	C20H18O7	370,1053	1500	No ionization	369	236(100), 270sh, 284sh, 310(90), 466(40)
Austocystin A	C19H13ClO6	372,0401	1169	373, 436	371	248(100), 290sh, 304(30), 340(14)

Alantrypinone	C21H16N4O3	372,1222	766	373, 228, 200, 436	371, 439, 226, 328, 313, 300,	212(100), 226(89), 258(27), 267(28), 278(22), 305(12), 317sh
Equisetin	C22H31NO4	373,2253	1360	374, 356, 346, 175, 188, 200, 294, 306	372, 342, 298	End(100), 236(38), 296(63)
Isomarticin	C18H16O9	376,0794	878	377, 359, 313, 287	375, 443, 331, 301, 274, 313, 287, 259	228(100), 304(30), 472sh, 500(27), 532sh
Marticin	C18H16O9	376,0794	900	377, 359 (PO)	375, 397, 331, 274, 443, 465	228(100), 304(33), 480sh, 500(29), 532sh
Verrucine A	C21H20N4O3	376,1535	774	377, 360, 332, 399, 440	375, 421, 284, 304	End(100), 230(79), 276(25), 308(10), 316sh
Dithiosilvatin	C18H22N2O3S2	378,1072	1072	379, 396, 401, 311, 251, 319, 220	377, 413, 345	End(100), 228(38), 272(22)
Hypothemycin	C19H22O8	378,1315	888	379, 361, 235, 253, 343, 207, 217, 401	377, 251, 413, 179, 163, 189, 207, 136	220 (100), 268(40), 308(19)
Fumitremorgin C; SM-Q	C22H25N3O3	379,1896	919	380, 324, 226, 412	378, 424, 803, 410, 458, 474	End(100), 224(95), 258sh, 272(22), 296(30)
Rotiorin	C23H24O5	380,1618	1371	337, 381, 444, 403	No ionization	End(55), 242(49), 285(32), 312(32), 404sh, 444sh, 47690), 502(100), 538sh
Benzomalvin D	C24H19N3O2	381,1477	1013	382, 423, 445, 249	No ionization	215(100), 232(100), 270(22), 282sh, 312(12), 324sh
Benzomalvin A	C24H19N3O2	381,1477	1078	382, 423, 351, 445, 323, 249	No ionization	215(100), 232(100), 270(22), 282sh, 312(12), 324sh
Monascorubramine	C23H27NO4	381,1940	1133	382, 445, 404, 338, 256	380, 365	262(70), 304(100), 416(88), 528(96)
Mitorubrin	C21H18O7	382,1053	1032	383, 296, 233, 215, 151	381, 399, 363, 337, 149, 123, 231, 249, 269	216(88), 268(100), 294sh, 350(88), 361sh
Neosolaniol	C19H26O8	382,1628	703	400, 405, 365, 446, 365, 335, 305, 275, 257, 245, 215	427, 417 (PO)	End
Monascorubrin	C23H26O5	382,1780	1422	383, 446, 405, 421, 339, 321	381, 337	222(30), 252(40), 290(35), 472(100)
T-2 Triol	C20H30O7	382,1992	812	383, 233, 215, 400, 281, 322	427, 417	End
Nidurufin	C20H16O8	384,0840	939	385, 367 (Very poor ioonization)	383	200(100), 224(60), 294(88), 456(30)
Stachybotrylactam	C23H31NO4	385,2253	992	386, 427, 449	384, 430	220(100), 266(20), 304(12)
Pitholide B	C22H26O6	386,1729	1142	387, 450, 425, 409	385	260
Ankaflavin	C23H30O5	386,2093	1407	387, 450, 409, 315	385	394
Atranone E	C24H34O4	386,2457	1129	387, 828	431, 421(PO)	End(100), 236(79)
Cholesterol	C27H46O	386,3549	2320	369 (VPO)	309, 377, 485 (VPO)	End
PF-1	C22H23N5O2	389,1852	900	390, 322	388	208(100), 228(60), 308(86)
PF-3	C22H23N5O2	389,1852	900	390, 322	388	208(100), 228(60), 308(86)
Roquefortine C	C22H23N5O2	389,1852	801	390, 322, 193	388, 434, 190	204(100), 236(43), 304(79)

Sclerotiorin	C21H23ClO5	390,1228	1416	303, 349, 391	No ionization	End(40), 220sh, 288(23), 298sh 260(100), 368(100), 392sh, 416sh, 446sh, 480sh
Compactin	C23H34O5	390,2406	1174	408, 391, 454, 413, 271, 185, 159, 229, 211, 253	435, 389 (PO)	228sh, 236(100), 242sh
Vermiculin	C20H24O8	392,1471	812	410, 393, 375	391, 195, 437, 427	224 nm
Asperloxin A	C21H19N3O5	393,1325	878	394, 435, 457, 379	392 (VPO)	208(100), 240(49), 260(13)
Circumdatin-A	C21H19N3O5	393,1325	897	394, 435, 457	392, 438, 377, 349	204(100), 236(53), 290sh, 356(16)
Ergocalciferol	C28H44O	396,3392	23	397, 379	No ionization	214(100), 268(100)
Ergosterol	C28H44O	396,3392	2136	395, 377, 379, 481 (VPO)	495 (VPO)	End(100), 272(100), 284(100), 290sh
Mitorubrinol	C21H18O8	398,1002	862	399, 249, 151, 192, 231, 312	397, 415, 353, 229, 149, 167	212(94), 268(100), 294sh, 352(88), 363sh
Harzianum A	C23H28O6	400,1886	1044	401, 277, 233, 215, 210, 151,	399, 445, 467	202(40), 306(100)
Mycelianamide	C22H28N2O5	400,1998	1146	401, 442, 265, 383	399, 200, 131	End(100), 232(32), 320(78)
Ophiobolin A	C25H36O4	400,2614	1128	401, 365, 383, 464, 347, 283, 239	399 (VPO)	End(60), 242(100)
Entonaemin A	C21H22O8	402,1315	826	403, 466, 235, 253, 151, 316	401, 357, 233, 167, 149 (PO)	218(100), 264(47), 302(20), 390(49)
Citreoviridin	C23H30O6	402,2042	971	403, 425, 461, 441, 315, 285	447 (VPO)	204(30), 236(20), 296(50), 388(100)
Desacetyl-fusaproliferin	C25H38O4	402,2770	1044	385, 403, 466	No ionization	End(100), 266(70)
Citreoviridin	C23H30O6	402,2042	968	403, 315,425, 461, 420, 297, 285	447 (VPO)	204(30), 236(20), 296(50), 388(100)
Ophiobolin B	C25H38O4	402,2770	1115	403, 385, 367, 466, 349, 293	401 (VPO)	End(60), 242(100)
Ochratoxin A	C20H18ClNO6	403,0823	1025	404, 358, 257	402, 358, 424, 211, 254, 314	216(100), 250sh, 332(20)
Ascochlorin	C23H29ClO4	404,1754	1382	405, 387, 199, 240, 281,	403	242(100), 294(40), 240(18)
Scytalidic acid	C22H28O7	404,1835	1299	387, 405, 428, 446, 465, 528	403, 359	212(100), 250(66)
Mevinolin-open lactone form	C24H36O5	404,2563	1233	422, 405, 427, 443, 468, 303, 285, 199, 225, 267, 173, 249	449 (PO)	238(100), 246sh(70)
16-epi-hydroxy-roquefortine C	C22H23N5O3	405,1801	795	406, 338, 320, 388	404, 540, 440, 386, 317	End(100), 246(14), 312(68)
16-hydroxy roquefortine C	C22H23N5O3	405,1801	777	406, 338, 320, 388, 428	404, 440, 450, 472, 386, 317, 206, 335	End(100), 246(14), 312(68)
Emindole DA	C28H39NO	405,3032	1513	406, 438, 447, 388, 259, 171, 130	404, 434, 436, 450	End(100), 224(96), 284(18), 290sh
Nominine	C28H39NO	405,3032	1601	388, 406, 271, 332, 447,	450, 434, 404,	End(100), 224(94), 284(18), 291sh
Shamixanthone	C25H26O5	406,1775	1844	389, 323, 470,	No ionization	End(100), 244(61), 276(82), 304(22), 396(20)
3,4,15-Triacetoxyscirpenol	C21H28O8	408,1784	954	426, 431, 472, 349, 307, 289, 247, 229	No ionization	End
Variecoxanthone B	C25H28O5	408,1931	1832	391, 323, 472, 409	No ionization	End(100), 244(61), 276(82), 304(22), 396(20)
Verrucofortine	C24H31N3O3	409,2365	1007	410, 300, 304, 532	No ionization	208(100), 230(75), 264(30), 334(48)
Verrucosine	C24H31N3O3	409,2365	1012	410, 473, 342, 300, 841	408, 454, 351	204(100), 248(31), 275sh

Desertorin A	C22H18O8	410,1002	876	411, 452	409	205(100), 210sh, 243sh, 297sh, 304(52), 308sh
Orlandin	C22H18O8	410,1002	839	411, 433, 474,	409, 477	212(100), 235(sh), 295(sh), 308(45), 325(sh)
Mitorubrinic acid	C21H16O9	412,0794	878	151, 192, 247, 263, 413	261, 205, 367, 411, 433	212(78), 272(100), 296sh, 448(65), 362sh, 393sh, 418sh, 448sh
Fucosterol	C29H48O	412,3705	2354	395, 454 (VPO)	235 (VPO)	End
AK Toxin I	C23H27NO6	413,1838	940	414, 436, 204, 222, 245, 263, 176, 134	412, 458, 480, 220, 202, 178	Edn(80), 216sh, 294(100)
TAN-1612	C21H18O9	414,0945	987	415, 397, 369, 415 (PO)	413, 435, 369, 285	230(64), 276(100), 322(24), 328sh, 408(37)
Tentoxin	C22H30N4O4	414,2267	888	415, 437, 302, 358, 312, 199, 171, 217	459, 413, 271, 369, 141, 214, 246	End(100), 218(55), 280(78)
Nimbosterol	C29H50O	414,3862	2454	383, 413, 431, 442, 454 (VPO)	No ionization	End
Cephalosporin C	C16H21N3O8S	415,1049	675	416, 438, 356, 312	414, 370, 354, 436	End
Cylindrospermopsin	C15H21N5O7S	415,1162	700	416, 457,	414, 436	End
Elymoclavine-O-β-D-fructofuranoside	C22H28N2O6	416,1947	662	417, 255, 237	461, 415	224(100), 282(21)
Atranone A	C24H32O6	416,2199	1012	458, 417, 399, 381, 355, 251, 233, 355	461, 415, 371, 327, 269, 285	End(100), 224sh(65)
Asteltoxin	C23H30O7	418,1992	902	419, 441, 259, 231, 277, 299	417, 463, 208	272(90), 368(100)
Citreoviridin	C23H30O7	418,1992	939	275, 419, 441, 693, 837, 859	463, 453	216(25), 272(81), 372(100)
13-Dehydroxypaxilline	C27H33NO3	419,2460	1360	420, 402, 362, 330	418, 464, 360,	234(100), 250sh, 284(23)
Epicorazine A	C18H16N2O6S2	420,0450	764	485, 421	483, 417, 529	End(100), 228sh(50)
Trichoverrol A	C23H32O7	420,2148	813	438, 443, 421, 403, 459, 249, 290, 231, 213	465, 474, 489 (PO)	200(50), 264(100)
Trichoverrol B	C23H32O7	420,2148	803	438, 443, 421, 403, 459, 249, 290, 231, 213	465, 474, 489 (PO)	200(50), 264(100)
Emindole DB	C28H39NO2	421,2981	1503	404, 422, 454, 463, 130, 171	420, 450, 452, 466	End(100), 224(96), 284(18), 290sh
Paspaline	C28H39NO2	421,2981	1617	422, 404, 275, 454, 438	420, 452, 144 (VPO)	207sh(48), 232(100), 280(29)
Mevinolin-open lactone form	C24H38O6	422,2668	1129	405, 445, 423, 321, 303, 285, 267, 199, 173	421, 467, 489, 319	238(100), 246sh(70)
Demethylkotanin	C23H20O8	424,1158	915	425, 447	423	210(100), 235(sh), 295(sh), 308(45), 325(sh)
Desertorin B	C23H20O8	424,1158	955	425, 488	423, 491	205(100), 210sh, 243sh, 297sh, 304(52), 308sh
HT-2 toxin	C22H32O8	424,2097	878	425, 442, 447, 463, 263, 245, 215	469, 459	End
Lanosterol	C30H50O	426,3862	2354	409, 441, 468 (PO)	No ionization	End
Wortmannin	C23H24O8	428,1471	750	429, 387, 446, 313, 369, 355, 295, 255	427, 399, 459, 417, 341, 385, 369, 356 (VPO)	End(100), 260(50), 296(32)
Insulicolide A	C22H25NO8	431,1580	919	414, 432, 449, 306 (VPO)	476, 166, 430, 412, 368	End(100), 212sh(75), 260(65)
Insulicolide B	C22H25NO8	431,1580	871	432, 449, 495, 414, 288, 247, 299, 217	476, 430, 466, 412, 166	End(100), 212sh(75), 260(65)
Pseurotin A	C22H25NO8	431,1580	817	334, 348, 432, 454, 495, 470, 316, 273	430, 270, 308, 354, 386, 476, 498	End(100), 258(69), 285sh

Fusarin C	C23H29NO7	431,1944	990	414, 432, 449, 384, 396, 454, 364	430, 476	258sh, 366(100)
6-epi-Stephacidin A	C26H29N3O3	431,2209	970	432, 219, 473, 387	No ionization	End(83), 210sh, 246(100), 310(22), 334sh
Stephacidin A	C26H29N3O3	431,2209	973	432, 219, 260, 473	430, 448, 390, 476	End(84), 208sh, 256(100), 310(30), 232sh
Chaetoviridin A	C23H25ClO6	432,1334	1271	433, 333, 389, 455, 417, 496	431, 387, 351	220(64), 310(100), 366(72), 424sh
Psychrophilin A	C23H20N4O5	432,1434	1052	450, 496. 433, 471, 304, 243, 130, 386	431, 384, 302, 255, 211	End(100), 250(30), 302 (13)
Atranone F	C24H32O7	432,2148	1052	433, 474, 373, 355, 337, 327, 309	477, 467, 417, 371, 413, 389, 353, 327	End(100), 232sh(50)
Meleagrine	C23H23N5O4	433,1750	767	434, 403, 334, 318, 289	432, 363, 332	End(100), 228(71), 284sh, 328(72)
Paspalinine	C27H31NO4	433,2253	1268	434, 376, 416, 466, 450	478, 432, 468, 374	End(60), 232(100), 273(27)
Notoamide E	C26H31N3O3	433,2365	1031	432, 378, 366, 280, 497, 212, 497	432, 478, 496, 468, 392, 153, 278, 240, 296	End(86), 214(78), 244(100), 310(29), 334sh
Chaetoviridin C	C23H27ClO6	434,1490	1160	417, 435, 498, 389,	433, 497	224(86), 294(54), 302sh, 288(100), 404(98), 426sh
Paxilline	C27H33NO4	435,2410	1227	436, 418, 400,	480, 434, 470, 376, 358, 330, 304	234(100), 282(20)
HC Toxin I	C21H32N4O6	436,2322	750	459, 437, 475, 497, 482, 409, 240	435 (PO)	End very poor UV sensitivity
16-Deoxopaxillin-16 -ol	C27H35NO4	437,2566	1069	420, 402, 438, 501, 470, 476, 420, 384,	478, 472, 436	End(80), 232(100), 284(28)
α-Paxitriol	C27H35NO4	437,2566	1116	438, 437, 470, 402, 420, 362, 344	478, 472, 436	230(100), 280(25)
20,25-Dihydroxyaflavinine	C28H39NO3	437,2930	983	420, 402, 438, 303, 285	436	End(91), 224(100), 284(19)
Desertorin C	C24H22O8	438,1315	1023	439, 480, 502	423, 355 (VPO)	205(100), 210sh, 243sh, 297sh, 304(52), 308sh
Kotanin	C24H22O8	438,1315	1012	439, 461, 477	No ionization	208(100), 235sh, 296sh, 308(47), 316sh
Mitorubrinol acetate	C23H20O9	440,1107	976	441, 291, 151, 192, 504	439, 395	216(90), 266(100), 294sh, 348(88)
Nidulin	C20H17Cl3O5	442,0142	1456	443, 407, 387, 484	441	204(100), 223sh(90), 268(18)
Hypomiltin	C23H22O9	442,1264	980	443, 460, 151, 192, 291,	401, 149, 167, 291	End(100), 210(92), 274(71), 310(88)
Andrastin E	C26H34O6	442,2355	1014	443, 506, 411, 383	441	End(100), 262(72)
Rugulosuvine B	C27H29N3O3	443,2209	1027	444, 485, 376	442, 488, 351	200(100), 248(32), 260sh
Rubiginosin	C23H24O9	444,1420	847	445, 277, 295, 235, 217, 151	443, 167, 233, 275, 123, 149, 247	220(100), 265(40), 302(18), 388(40)
Fusaproliferin	C27H40O5	444,2876	1181	427, 445, 462, 508	No ionization	End(100), 264(58)
Dystanine	C30H52O2	444,3967	2324	440, 381, 255 (VPO)	497, 526 (VPO)	End
Alternarolide I	C23H31N3O6	445,2213	910	463, 446, 468, 258, 161, 418	490, 444, 558, 400, 304	End(100), 222(54), 245sh, 275sh
Dihydrofructigenine A	C27H31N3O3	445,2365	1053	446, 376, 334, 568, 487, 509	444, 490	204(100), 248(30), 280sh
Atranone B	C25H34O7	446,2305	1078	447	445, 491	End(100), 230sh(65)

Oxaline	C24H25N5O4	447,1907	786	448, 417, 348, 332, 386, 402, 305	446, 384, 362, 346, 330, 318	End(100), 228(70), 284sh, 328(72)
Notoamide B	C26H29N3O4	447,2158	951	489, 448, 895, 466, 420	446, 464, 406	End(90), 248(100), 274sh, 314sh
Versicolamide B	C26H29N3O4	447,2158	911	448, 895	446, 464, 406, 478	End(87), 248(100), 274sh, 316(4)
Marcfortine C	C27H33N3O3	447,2522	771	448, 466, 420, 895, 489,	446, 464, 406	End(58), 248(100), 275sh, 318(4)
Psychrophilin D	C24H24N4O5	448,1747	1106	466, 449, 249, 320, 290	447, 318, 271	End(200), 216(86), 244(70), 304(23)
Atranone H	C24H32O8	448,2097	1015	403, 431, 449, 490	447, 493	End
S14-95	C28H34O5	450,2406	1472	451, 242, 391, 201, 191	No ionization	204(100), 240(61), 324(50)
Cytochalasin J	C28H37NO4	451,2723	580	452, 434, 416, 398, 475, 392	496, 486, 450, 432, 340	End
Dehydroaustinol	C25H28O8	456,1779	962	498, 457	501, 455	End(100), 248(54)
Communesin A	C28H32N4O2	456,2525	1072	457, 479, 385	*501, 491, 485, 469, 399	204(100), 250(18), 268(20)
DON-3-glucoside	C21H30O11	458,1788	681	476, 481, 297 (PO)	503, 427, 457, 409	220
Austinol	C25H30O8	458,1935	939	500, 459	503, 457	End(100), 248(54)
trans-Fumagillin	C26H34O7	458,2305	1109	476, 459, 427, 522, 409, 177, 215, 233	503, 457, 525, 413	End(85), 232(100)
Aurovertin B	C25H32O8	460,2097	1054	461, 261, 502, 478, 502, 183, 141, 231	505, 145 (PO)	274(98), 368(100)
Lumpidin	C26H28N4O4	460,2111	880	461, 443, 239	459, 505,	End(100), 250, 285sh
Echinulin	C29H39N3O2	461,3042	1307	462, 479, 484, 406, 338, 525, 394, 326, 266, 334	383, 460, 423	End(98), 232(100), 280(24)
AM Toxin I- open lacone form	C23H33N3O7	463,2319	897	464, 306, 486, 375, 278	462	End(100), 222(52), 250sh, 274sh
Iso-T-2 Toxin	C24H34O9	466,2203	1005	484, 467, 489, 530, 215, 245, 227, 287, 305, 387	511, 501 (PO	End
T-2 toxin	C24H34O9	466,2203	980	484, 530, 489, 335, 215, 245, 305, 346, 368, 387	511, 501 (PO)	End
Penigequinolone A	C27H33NO6	467,2308	1167	450, 418, 468, 436, 310, 296, 278	466	End(199), 222&63), 230sh, 280(33), 288sh, 326(32)
Nebramycin 6	C18H37N5O9	467,2591	670	468	No ionization	Not deteceted
Communesine G	C29H34N4O2	470,2682	922	471, 493, 399, 501	No ionization	End(100), 272(7)
1'-O-Acetylpaxilline	C29H35NO5	477,2515	1353	478, 477, 418, 462, 477, 400, 510, 346,	476, 522, 416,	234(100), 284(20)
Cytochalasin A	C29H35NO5	477,2515	1085	478, 460, 442, 500, 541, 414, 394	476, 522, 458, 432 (PO)	End, 230sh
Fumitremorgin B	C27H33N3O5	479,2420	1158	462, 543, 507, 480, 512, 281, 394, 406	478 VPO	End(100), 228(94), 278(18), 296(21)
Cytochalasin B	C29H37NO5	479,2672	945	480, 462, 444, 426, 416, 543	524, 478, 514, 460, 500	End
Distamycin A	C22H27N9O4	481,2186	680	482, 291, 395, 273, 247	480	200(75), 236(80), 308(100); HCl: (235, 470) (302, 565)
Dihydrocytohalasin B gamma lactone	C29H39NO5	481,2828	1014	482, 446, 464, 504, 428, 410, 520	526, 480, 516, 462, 280, 214, 378	End
Verrucarin J	C27H32O8	484,2097	1162	502, 485, 507, 523, 231, 249, 213	501, 483, 529 (VPO)	220(100), 264(71)
Kanamycin	C18H36N4O11	484,2381	670	485	No ionization	Not detected
Dimerum acid	C22H36N4O8	484,2533	673	485, 507	483	End
Ferri-dimerum acid	C22H34FeN4O8	484,2533	672	538, 579	536	Not detecetd
Communesine H	C30H36N4O2	484,2838	991	485, 515, 413, 507	No ionization	End(100), 272(7)
Staplabin	C28H39NO6	485,2777	1118	486, 468, 508	530, 484, 552, 530, 542	216(100), 256(21), 302(6)

Andrastin A	C28H38O7	486,2618	1105	487, 427, 509, 409, 395, 377, 367	485, 453, 425	End(100), 262(53)
Pyrrocidine A	C31H37NO4	487,2723	1597	488, 283, 470, 434	486, 532	End(100), 236sh, 276(3)
Terretonin	C26H32O9	488,2046	994	489, 506, 471, 453	487, 255, 427, 533, 523	End(78), 280(100)
Pyrrocidine B	C31H39NO4	489,2879	1423	490, 283, 472, 255	488, 534, 502	End(100), 232(21), 276(3)
Paraherquamide A	C28H35N3O5	493,2577	762	494, 476, 466, 419, 352	492	228(100), 256sh, 278sh
Paspalin P1	C30H39NO5	493,2828	946	434, 476, 494, 416, 398, 557, 392	540, 538, 598 (VPO)	End
Cytochalasin E	C28H33NO7	495,2257	1019	513, 518, 496, 434, 534, 550, 478, 416, 336, 204, 258,	540, 494, 432, 450, 414, 360, 282, 269 (PO)	End
Aflatrem	C32H39NO4	501,2879	1498	502, 444, 198	546, 400, 442 (PO)	232(100), 284(30)
Verrucarin A	C27H34O9	502,2203	975	503, 520, 525, 457, 249, 231, 290, 555, 566	547, 537, 501, 455, 389 (VPO)	260
Tryptoquialanine B	C26H24N4O7	504,1645	994	505, 487, 443, 568, 543, 383, 233, 183	549, 539, 503, 443, 461, 425, 145, 189, 225	208(100), 234(74)
AAL Toxin TB1	C25H47NO9	505,3251	804	506, 528, 488, 312, 330, 294	504, 526	Cannot be detected using UV
Asperphenamate	C32H30N2O4	506,2206	1202	507, 529, 256, 238, 224	551, 268, 505	End(100), 228sh
1,1-Bis(6,8-dimethyl-8,9-epoxy-5a,10e)ergoline	C32H34N4O2	506,2682	698	507, 254, 274, 295	No ionization	222(100), 274(33), 283sh, 295sh
Cytochalasin C	C30H37NO6	507,2621	1014	525, 508, 490, 430, 412, 265	552, 506 (PO)	End
Cytochalasin D	C30H37NO6	507,2621	947	525, 430, 490, 530, 508, 546, 412, 402, 384, 374	No ionization	End
Acetyl T-2 toxin	C26H36O10	508,2308	1118	531, 547, 572, 526, 197, 287	No ionization	End
Communesin B	C32H36N4O2	508,2838	1060	509, 531, 437, 451	553 (VPO)	204(100), 272(90)
Territrem A	C28H30O9	510,1890	1058	511, 593, 533	509, 395 (PO)	220(100), 340(48)
Alteramide A	C29H38N2O6	510,2730	1065	511	509, 511 PO	End
Verruculogen	C27H33N3O7	511,2319	1086	494, 575, 512, 410, 392, 352, 338,	510, 556, 426, 306, 350, 157 (PO)	230(100), 276(38), 300(34)
Verrucarin H	C29H36O8	512,2410	1247	530, 513, 535, 576, 551, 231, 249, 359	No ionization	226(100), 262(70)
Roridin E	C29H38O8	514,2567	1120	532, 537, 515, 553, 361, 231, 249, 267	515, 559, 549 (PO)	224(100), 264(80)
Malformin A2	C22H37N5O5S2	515,2236	905	533, 538	514 (PO)	End
Fusidic acid	C31H48O6	516,3451	1224	457, 439, 421, 534, 580, 520	515, 561, 455, 393, 221, 473, 411	End(100), 220sh(65)
Bisdethiodi(methylthio)acetylapoaranotin	C24H26N2O7S2	518,1181	1307	536, 541, 411, 303, 363, 381, 471	No ionization	End(100), 268(28)
Rubratoxin B	C26H30O11	518,1788	1013	536, 519, 501, 560, 582, 483	517, 403, 563, 473, 553, 535, 631, 359, 243	204(100), 252(32)
Tryptoquialanine A	C27H26N4O7	518,1801	1041	519, 457, 501, 582, 557, 397, 197, 213	563, 553, 517, 475, 401, 431, 457	214(100), 228(98), 280sh, 36sh
Rubratoxin A	C26H32O11	520,1945	875	538, 521, 485, 503, 389, 467	519, 555, 405, 475, 361, 457, 541, 317	204(100), 254sh(15)
AAL-Toxin TA	C25H47NO10	521,3200	767	522, 544, 346, 322, 310, 292	520	ND
1-Oleoyl-sn-glycero-3-phosphocholine	C26H52NO7P	521,3481	1234	522, 544	566, 506	Not detected
19,20-Epoxycytochalasin C	C30H37NO7	523,2570	966	524, 541, 464, 446, 428, 404, 386	568, 554, 522, 462	End
19,20-Epoxycytochalasin D	C30H37NO7	523,2570	900	524, 541, 464, 428, 446, 404, 386	568, 558, 522	End
Territrem B	C29H34O9	526,2203	1036	527, 509, 549	411, 525, 571, 491, 364	220(100), 332(48)

Hypocrellin B	C30H24O9	528,1420	1262	529, 592, 315, 405, 471, 271	527, 513, 595,	244(100), 214sh, 262sh, 336(10), 460(50), 548(25)
Satratoxin H	C29H36O9	528,2359	900	529, 546, 511, 592, 551, 511, 231, 249, 263	527, 573, 563, 483, 543 (PO)	200(85), 232(100), 265sh
Chaetoglobosin A	C32H36N2O5	528,2624	1048	546, 529, 551, 511, 382, 400, 382, 457	573, 527, 563, 484, 509, 466	End(90), 220(100), 276(17)
Chaetoglobosin C	C32H36N2O5	528,2624	1112	546, 529, 551, 567, 456, 259, 215, 346, 478	573, 527, 563, 509, 326, 380, 484, 455	220(100), 252(25), 280sh,290sh
Malformin A1	C23H39N5O5S2	529,2393	960	547, 552, 530, 568, 593, 502, 485, 417, 441, 425	528 (PO)	End, very poor sensitivity in UV
Malformin-C	C23H39N5O5S2	529,2393	983	547, 530, 552, 568, 593, 502, 485, 417, 372	574, 528	End
Roridin L-2	C29H38O9	530,2516	882	548, 531, 513, 283, 249, 231	575, 529, 455, 419, 375	End(85), 260(100)
Stemphone	C30H42O8	530,2880	1316	531, 513, 553, 594, 489, 471, 453, 429, 387,	529, 469,	End(100), 268(50), 296(5)
Dethiosecoemestrin	C27H20N2O10	532,1118	1515	406, 388, 438, 456, 479	434, 450, 468, 504	End(100), 224(91), 284(14)
Fumitremorgin D	C28H28N4O7	532,1958	1072	515, 533, 471, 261, 183, 211, 411, 455	577, 531, 531	208(100), 228(90), 255sh, 274sh, 308(8), 318sh
Roridin A	C29H40O9	532,2672	965	550, 555, 403, 249, 231	577 (PO)	264
Trichoverrin A	C29H40O9	532,2672	898	550, 533, 361, 515, 555, 231, 249, 571	577 (PO)	224(88), 260(100)
Trichoverrin B	C29H40O9	532,2672	899	550, 555, 533, 571, 515, 361, 249, 231, 213	577 (PO)	224(88), 260(100)
Didethiobis(methylthio)acetyl-aranotin	C24H26N2O8S2	534,1131	980	557, 552, 487, 397, 367, 319	No ionization	End(100), 225(79), 258sh
Rubratoxin-B-open form	C26H32O12	536,1894	884	554, 519, 537, 501, 483	535, 603, 491, 473, 403, 377, 447	208(100), 264sh
Cladofulvin	C30H18O10	538,0900	1165	539 (VPO)	537, 605, 559	End(80), 232(95), 268(100), 294sh, 448(44)
Skyrin	C30H18O10	538,0900	1273	539, 521 (VPO)	537	224(100), 256(81), 296(52), 456(40)
Rubratoxin A-open form	C26H34O12	538,2050	792	556, 539, 521, 389, 485	537, 519, 559, 475, 361, 405, 431, 317, 255	204(100), 254sh(15)
Rugulosin	C30H22O10	542,1213	1077	543, 279, 273, 584 (PO)	541, 563, 269, 471	End(88), 252(100), 274sh, 391(100)
Rubellin D	C30H22O10	542,1213	995	560, 525, 566, 507, 457, 606, 479 (PO)	541, 587, 563, 577, 523, 378, 360	216(100), 236(95), 252sh, 300(23), 498(27)
Isosatratoxin F	C29H34O10	542,2152	946	560, 543, 606, 525, 231, 249	541, 577, 587, 511, 189, 219, 481, 145, 391	200(100), 252(55)
Purpurugenone	C29H20O11	544,1006	1109	545 (PO)	543, 611, 527, 285, 565, 525, 499, 491, 581	222(100), 250(72), 276sh, 310(28), 380(26), 496sh, 520(22), 556sh
Satratoxin G	C29H36O10	544,2308	883	562, 545, 527, 608, 567, 231, 249	589, 499	200(100), 256(66)
Duclauxin	C29H22O11	546,1162	1080	564, 588, 547, 515, 455, 259 (PO)	545, 613, 513, 471	204(64), 228(100), 264sh, 320(13), 340sh
Ergosine	C30H37N5O5	547,2795	771	548, 530, 268	546, 296	End(100), 224sh, 240sh, 320(26)
Ergosinine	C30H37N5O5	547,2795	776	548, 530,	546, 296	End(100), 210sh, 242(52), 318(22)

Antimycin A1b	C28H40N2O9	548,2734	1500	549, 571, 265, 237	547, 245, 263	228(100), 320(18)
Manzamine A	C36H44N4O	548,3515	836	549, 531, 295, 275, 266, 286	593, 583, 547	216(100), 232sh, 260sh, 292(42), 308(35), 354(21)
Dihydroergosine	C30H39N5O5	549,2951	775	550, 532, 591, 270	548	224(100), 282(21)
Viomellein	C30H24O11	560,1319	1135	529, 592	559, 627, 581, 527, 627	228(48), 275(100), 360(18), 380(18), 415(17)
Ergocornine	C31H39N5O5	561,2951	786	562	560, 310	End(100), 224sh, 240sh, 322(26)
Ergocorninine	C31H39N5O5	561,2951	798	562, 544	560, 310	206(100), 224sh, 240(72), 318(26)
Viridicatumtoxin	C30H31NO10	565,1948	1175	548, 438, 565, 566, 531, 478	564, 586, 546	240(70), 284(100), 332(10),436(30)
Helvolic acid	C33H44O8	568,3036	1188	509, 586, 632, 572, 491, 463, 449, 439, 431, 403	567, 613, 507, 403, 525, 481, 463	End(100), 234(96)
Aurofusarin	C30H18O12	570,0798	1037	571, 593	569, 615	End(82), 248(100), 272(77), 380(20), 420(18)
Aurasperone A	C32H26O10	570,1520	1245	571, 634,	569	238(25), 282(100), 330(sh), 404(15)
Cyclochlorotine	C24H31Cl2N5O7	571,1601	784	572, 554, 485, 443, 338, 251, 218	534, 606, 616, 570, 468, 281, 387, 480, 498	End
SMTP-6	C34H40N2O6	572,2886	1205	573, 595, 527	617, 517, 607, 639,	216(100), 260(20), 284sh,292sh,206sh
Xanthomegnin	C30H22O12	574,1111	1020	No ionization	573, 595, 543, 527	232(100), 288(30), 403(20)
α -Ergocryptine	C32H41N5O5	575,3108	807	576, 558, 268, 305, 348	324, 574	End(100), 220sh, 240sh, 324(24)
α-Ergocryptinine	C32H41N5O5	575,3108	822	576, 558	574, 324	End(100), 210sh, 242(47), 318(23)
Destruxin A	C29H47N5O7	577,3475	940	578, 600, 465, 437	576, 622, 612, 504, 191, 225, 173, 353, 402, 424	End
Fumitremorgin A	C32H41N3O7	579,2945	1355	494, 562, 580, 602, 643, 478, 352, 392, 410, 420	No ionization	End(100), 228(94), 278(18), 296(21)
Ergotaminine	C33H35N5O5	581,2638	789	582	580, 330	End(100), 208sh, 240(46), 318(20)
Pyripyropene A	C31H37NO10	583,2417	985	584, 625	No ionization	232(100), 264(25), 320(70)
Dihydroergotamin	C33H37N5O5	583,2795	791	584, 566, 270, 253, 322	582, 467, 423, 313, 538	End(100), 224(80), 228(17)
Janthitrem B	C37H47NO5	585,3454	1075	586, 568, 510, 296	584, 630, 620, 676	End(80), 264(100), 332(60)
Baccatin III	C31H38O11	586,2414	893	527, 587, 650 , 604, 509, 405, 345, 327, 527	631, 621, 585, 543	End(100), 234(38), 276(4), 284sh
Aurasperone E	C32H28O11	588,1626	1100	589, 652, 453, 415	587, 655	238(30), 282(100), 330(sh), 402(15)
Emestrin A	C27H22N2O10S2	598,0716	956	599, 535, 517, 499, 489, 471, 245	597	End(100), 230sh, 260sh, 282sh
Penitremone A	C37H45NO6	599,3247	1265	600, 514, 532, 582	558, 644	260(100), 286sh, 336sh

Aurasperone B	C32H30O12	606,1732	1035	607, 589, 629, 670, 549, 645	605, 627, 573, 521	238(55), 282(100), 330(25), 334(25), 408(15)
Ergocristinine	C35H39N5O5	609,2951	720	610, 592, 305, 348, 223, 325, 268, 632, 648	608, 358	End(100), 210sh, 240(47), 318(20)
Lolitriol	C37H49NO7	619,3509	1140	620, 510	618, 664, 654	End(38), 264(100), 288sh, 338sh
Apicidin	C34H49N5O6	623,3683	1120	624, 641, 646	668, 622, 658, 462, 592	202(100), 224(92), 275sh, 292(13)
Rhizoxin	C35H47NO9	625,3251	1115	626, 596, 648, 664, 232, 284	670, 660 (PO)	297(79), 312(100), 326(75)
Enniatin B2	C32H55N3O9	625,3938	1370	643, 648, 214, 196, 598, 513, 413	No ionization	End
Cycloaspeptide D	C35H41N5O6	627,3057	1035	628, 650, 233, 297, 352, 368, 438, 451	626	End(100), 220(61), 258(29), 306(11)
Penitrem A	C37H44ClNO6	633,2857	1261	634, 616, 558	632, 678 (PO)	236(100), 300(30)
Secalonic acid D1	C32H30O14	638,1636	1088	639	637, 705 (PO)	End(82), 216(62), 237(48), 264(40), 337(100), 383sh
Secalonic acid D2	C32H30O14	638,1636	1164	656, 661, 639, 621, 579, 561	637, 705 (PO)	End(82), 216(62), 237(48), 264(40), 337(100), 383sh
Secalonic acid D3	C32H30O14	638,1636	1192	639, 661, 656, 677, 621, 579, 589, 561	637, 705 (PO)	End(82), 216(62), 237(48), 264(40), 337(100), 383sh
Enniatin B2	C33H57N3O9	639,4095	1418	657, 640, 662, 196, 527, 214, 427, 445	638, 610, 542, 425, 381	End
Enniatin-B1	C34H59N3O9	653,4251	1484	671, 654, 196, 210, 541, 627, 228, 441, 427	No ionization	End
Viriditoxin	C34H30O14	662,1636	1184	663	661	End(45), 224(40), 260(100), 342sh, 376(23)
Enniatin A1	C35H61N3O9	667,4408	1547	685, 668, 690, 210, 541, 196, 228, 441, 555, 459	No ionization	End
Neocoprogen II	C27H41FeN6O11	681,2183	672	682, 704	680	Not detected
Enniatin A	C36H63N3O9	681,4564	1611	699, 682, 704, 210, 555, 228, 455	No ionization	End
Enniatin C	C36H63N3O9	681,4564	1595	699, 682, 704, 210, 555, 228	No ionization	End
Lolitrem B	C42H55NO7	685,3979	1512	686, 620, 602, 584, 576	684, 730	End(38), 264(100), 288sh, 338sh
Fumonisin B4	C34H59NO13	689,3986	916	690, 712, 514, 496, 320, 338	688, 710	Not detected
Fumonisin B2	C34H59NO14	705,3936	880	706, 728, 688, 512, 336, 354	704	ND
Fumonisin B3	C34H59NO14	705,3936	857	706, 728, 788, 410, 334	704, 726, 750	Not detected
Chaetomin	C31H30N6O6S4	710,1110	1050	711, 728, 647, 348, 298, 364	645, 709, 579	End(100), 218sh, 286sh(13)
Fumonisin B1	C34H59NO15	721,3885	826	722, 744, 704, 642, 546, 352, 528	720, 742, 764	ND
Fumonisin B6	C34H59NO15	721,3885	868	722, 546, 370, 352	720, 742	Not detected
Nigericin	C40H68O11	724,4762	1924	742, 675	723	End
Neocoprogen I	C31H47FeN6O12	751,2601	683	752	796, 750	Not detected
Ferrichrome C	C28H44FeN9O12	754,2459	677	755, 772, 777	753, 799, 789	Not detected
Fusigen	C33H51FeN6O12	779,2914	672	390.6 (db), 780, 802	PO	End(100), 210(100), 250sh, 430(14)
Beauvericin	C45H57N3O9	783,4095	1521	801, 784, 806, 750, 685, 668, 244	782, 431	End(100), 210sh
Phalloidin	C35H48N8O11S	788,3163	741	789, 753, 771, 725, 811	787. 833, 1577, 1623	End(100), 220(80), 292(30)
Cladochrome E	C44H38O14	790,2262	1303	791, 813, 515, 854, 483, 637, 829, 669	789, 857, 811, 773	224(100), 268(50), 476(41), 543(20), 584(20)

Coprogen	C35H53FeN6O13	821,3020	686	822, 844	866, 820	Not detected
Rutilin A	C44H40O16	824,2316	1188	825, 847, 675, 657, 507, 489	823, 655, 487, 167, 673	216(100), 268(73), 306(48), 415sh, 464(56)
Triacetylfusarinine C	C39H57N6O15	849,3898	777	906, 928	950, 904	End(100), 210(100), 250sh, 450(12)
Taxol	C47H51NO14	853,3310	1083	854, 776, 836, 876, 569, 551, 509, 387, 286, 268, 240	898, 888, 852, 792, 571, 525, 449, 403	End(100), 232(43)
SMTP-7	C51H68N2O10	868,4874	1435	869, 891,	867, 935, 903, 841, 773	216(100), 260(22), 304(8)
SMTP-8	C52H70N2O10	882,5030	1485	884, 857, 789	881, 949, 787, 855	216(100), 256(20), 302(8)
Amphotericin B	C47H73NO17	923,4879	874	924, 906, 743, 946, 761, 725, 707	922 (PO)	228(10), 340(25), 364(52), 384(92), 408(100)
Aculeacin A	C50H81N7O16	1035,5740	1119	1018, 1058, 1036, 1000, 874, 892, 762, 568	990, 1034, 946, 794, 300, 566, 469, 539	End(100), 240sh, 276(4)
Valinomycin	C54H90N6O18	1110,6312	2090	1128, 1149	1155, 1109, 1223, 956, 928, 757	End (Poor sensitivity in UV)
Cereulide	C57H96N6O18	1152,6781	2172	1170, 1175	1151, 1187, 970, 984, 785	Not detected
Homocereulide	C58H98N6O18	1166,6938	2269	1184,5	1165, 1201, 1228, 984, 970, 998	Not detected
Cyclosporin A	C62H111N11O12	1201,8414	1400	1202, 1224, 1219	1246, 1200	End
Trichofumin-B	C60H106N12O13	1202,8002	1300	1225.5, 1203.5, 989, 581, 623, 496, 409, 383	1201.6, 1247.6	End
Trichofumin-BX	C60H109N13O12	1203,8319	1327	1225.5, 1203.5, 989, 581, 623, 496, 409, 383	1202.6, 1270.5	End
Cyclosporin-C	C62H111N11O13	1217,8363	1420	1219, 1241, 1236, 610(db),	1262.7, 1216.7, 1330.7, 1172.7	End
Trichofumin C	C69H120N16O17	1444,9017	1015	215, 1231, 936, 454, 616, 745, 1467.9, 1445.9	1489.9, 1443.9	End
Ampullosporin	C77H127N19O19	1621,9556	2167	*758, 906, 503, 429, 322, 239	No ionization	220(100), 280broad(15)
Efrapeptin F	C82H141N18O16	1634,0767	1007	1633, 931, 703, 350, 1284	1631, 815(db)	End
Antiamebin I	C82H127N17O20	1669,9443	1066	1671, 249, 544, 787, 884, 1422, 1224, 1693	1669, 1715, 883 (db), 856(db)	End
Trichotoxin A50E	C78H136N20O21	1689,0189	1252	612, 851, 1078, 839, 1690, 766, 438	1687.8, 1733.8	End
Paracelsin A	C88H145N23O24	1908,0833	1123	774, 1135, 908, 1001, 955, 966, 1909	1907, 960, 967, 1953	End
Paracelsin C	C89H147N23O24	1922,0989	1179	774, 1149, 962, 922, 1001, 788, 1923, 1945	1921, 960, 1967	End(100), 222sh
Trichocellin A	C89H147N23O24	1922,0989	1138	774, 1149, 922, 962, 1101, 973, 1923, 1945	1921, 960, 1967	End(100), 222sh
Paracelsin D	C90H149N23O24	1936,1146	1182	774, 1163, 1149, 969, 1001, 936, 1937, 1959	1935, 967, 1981	End(100), 222sh
Alamethicin F	C92H151N23O24	1962,1302	1275	1963, 1189, 774, 934, 982, 1029	1961, 980(db)	End
Atroviridin A	C92H151N23O24	1962,1302	1257	774, 1189, 934, 1963, 1029	1961	End
Atroviridin B	C93H153N23O24	1976,1459	1290	788, 1189, 934, 1977, 810, 774	1975	End
Polysporin B	C93H153N23O24	1976,1459	1324	774, 1203, 948, 1977, 796, 1029, 989, 1999, 1000	1975	End
Atroviridin C	C94H155N23O24	1990,1615	1358	788, 1203, 810, 1991, 948, 1007, 1043	1989	End

**(2M-2H+Fe3) PO poor ionization

***(MW not to be determined) VPO very poor ionization

* cheats

db (doubly charged) **** low amount of compound avaliable ***** difficult to tetermine if water addition happens in column or ESI-	MI mono isotopic mass RI Retention index relative to alkylphenones					

Table 3 common "Jumps" (Δ) observed in Electro spray

Positive electrospray

Adducts relative to $[M+H]^+$	Δ (amu) [a]
Na^+, very stable adduct [b]	0,0000
NH_4^+ [c]	17,0265
MeCN, neutral adduct [d]	41,0265
Na^+ + MeCN , very stable adduct [b, d]	41,0265
K^+ , very stable adduct [b]	0,0000
K^+ + MeCN , very stable adduct [b, d]	41,0265
$2Na^+$ - H (acids, phenols and enoles)	0,0000
Fe^{2+} , isotope m/z 2 lower ca. 4 % [e]	0,0000
Exchanging adducts	
Na^+ instead of NH_4^+	-17,0265
K^+ instead of Na^+	0,0000
$[M - H_2O + H]^+$ to $[M + Na]^+$	18,0106
$[M - H_2O + H]^+$ to $[M + MeCN + Na]^+$ [d]	59,0371
$[M - H_2O + H]^+$ to $[M + NH_4]^+$	35,0371
$[M - 2 \times H_2O + H]^+$ to $[M + Na]^+$	36,0212
$[M + NH_4]^+$ to $[M + H - CH_3COOH]^+$ [d]	77,0476
$[M + Na + MeCN]^+$ to $[M + H - CH_3COOH]^+$ [d]	101,0476
Neutral losses	
H_2O	18,0106
CO_2	43,9898
CH_3CHO	44,0262
HCOOH	46,0055
CH_3COOH	60,0211
NH_3 (rare) [c]	17,0265

Negative electrospray

Adducts relative to $[M-H]^-$	Δ (amu) [a]
HCOOH [f]	46,0055
CH_3COOH [f]	60,0211
Na^+ -H (acids, phenols and enoles)	21,9820
Cl^- (A+2 isotope)	35,9767
Opening of lactone etc. (water addition)	18,0106
M-2H+HCOOH+Na	67,9874
Exchanging adducts	
HCOOH instead of Cl^-	10,0288
Neutral losses	
H_2O	18,0106
CO_2	43,9898
CH_3	15,0235
CH_3CHO	44,0262
HCOOH	46,0055
CH_3COOH	60,0211

[a] mass shift

[b] increased by high in-source fragmentation settings

[c] loss or addition of NH_3 can usually be determined by looking after the $[M+Na]^+$ ion

[d] if MeCN is used as solvent. MeCN forms NH_3 upon acdic hydrolysis

[e] sideophors and artefact from Fe^{2+} liberation ESI^+

[f] if added to solvent

Table 8	Combined positive and negative ionization electrospray to determine correct molecular mass						
Ionization					$[M+H]^+$ & $[M-H]^-$	$[M+NH_4]^+$ & $[M-H+HCOOH]^-$	ESI^+ combined with $[M-H]^-$ is unambiguous	92.9
Both poor (%)	2.7	Most intense both (%)			37.4	3.5	ESI^+ alone is unambiguous	56.4
None both (%)	1.6	Observed both (%)			60.1	9.7	ESI^- alone is unambiguous	36.5

Paper 3

"Bioactivity, Chemical Profiling, and 16S rRNA Based Phylogeny of *Pseudoalteromonas* Strains Collected on a Global Research Cruise"

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ORIGINAL ARTICLE

Bioactivity, Chemical Profiling, and 16S rRNA-Based Phylogeny of *Pseudoalteromonas* Strains Collected on a Global Research Cruise

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Abstract One hundred one antibacterial *Pseudoalteromonas* strains that inhibited growth of a *Vibrio anguillarum* test strain were collected on a global research cruise (Galathea 3), and 51 of the strains repeatedly demonstrated antibacterial activity. Here, we profile secondary metabolites of these strains to determine if particular compounds serve as strain or species markers and to determine if the secondary metabolite profile of one strain represents the bioactivity of the entire species. 16S rRNA gene similarity divided the strains into two primary groups: One group (51 strains) consisted of bacteria which retained antibacterial activity, 48 of which were pigmented, and another group (50 strains) of bacteria which lost antibacterial activity upon sub-culturing, two of which were pigmented. The group that retained antibacterial activity consisted of six clusters in which strains were identified as *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas aurantia*, *Pseudoalteromonas phenolica*, *Pseudoalteromonas ruthenica*, *Pseudoalteromonas rubra*, and *Pseudoalteromonas piscicida*. HPLC-UV/VIS analyses identified key peaks, such as violacein in *P. luteoviolacea*. Some compounds, such as a novel bromoalterochromide, were detected in several species. HPLC-UV/VIS detected systematic intra-species differences for some groups, and testing several strains of a species was required to determine these differences. The majority of non-antibacterial, non-pigmented strains were identified as *Pseudoalteromonas agarivorans*, and HPLC-UV/VIS did not further differentiate this group. *Pseudoalteromonas* retaining antibacterial were more likely to originate from biotic or abiotic surfaces in contrast to planktonic strains. Hence, the pigmented, antibacterial *Pseudoalteromonas* have a niche specificity, and sampling from marine biofilm environments is a strategy for isolating novel marine bacteria that produce antibacterial compounds.

Keywords *Pseudoalteromonas* · Antibacterial activity · Secondary metabolites · Bioprospecting · Galathea 3

Introduction

Compounds of relevance for the pharmaceutical and biotechnology industries are produced by marine microorganisms (Burgess et al. 1999), and it has been suggested that some compounds of pharmacological interest previously attributed to macroorganisms may in fact be of microbial origin (Bewley and Faulkner 1998; Simmons et al. 2008; Sudek et al. 2006). The emergence of multiresistant pathogenic bacterial strains and the failure of combinatorial and diversity-oriented chemistry to adequately supply the drug discovery pipeline (Newman 2008) have re-invigorated natural product chemistry as a path for discovery and development of new antibiotics. With this in mind, we isolated marine bacteria with antibacterial activity during the Danish Galathea 3 marine research expedition (Gram et al. 2010). The antibacterial strains were tentatively identified using 16S rRNA similarity, and one of the major groups of

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isolated bacteria was identified as *Pseudoalteromonas* (Gram et al. 2010).

The genus *Pseudoalteromonas* consists of Gram-negative marine bacteria belonging to the γ-proteobacteria and is present globally in marine waters where they constitute 0.5% to 6% of the total bacterioplankton (Wietz et al. 2010). They are heterotrophic aerobes and non-fermentative, and the cells are motile by one or more polar flagella. The genus divides into two groups: pigmented and non-pigmented species. The pigmented species are often producers of bioactive secondary metabolites (Bowman 2007) displaying cytotoxic (Zheng et al. 2006), antibacterial (Gauthier 1976b; Gauthier and Flatau 1976; Isnansetyo and Kamei 2003; Jiang et al. 2000; McCarthy et al. 1994), antifungal (Franks et al. 2006; Kalinovskaya et al. 2004), or antifouling (Egan et al. 2001; Holmström et al. 2002) effects. It has been hypothesized that bioactive *Pseudoalteromonas* are primarily associated with higher organisms (Holmström and Kjelleberg 1999), suggesting an ecological role in which some bioactive species might play an active part in host defense against pathogens and fouling organisms (Holmström et al. 1996; Armstrong et al. 2001; Egan et al. 2008). A link between surface colonization and antibacterial activity has not been experimentally verified, although several studies have successfully isolated epiphytic bacteria with antibacterial activity from algae and other marine organisms (Armstrong et al. 2001; Boyd et al. 1999; James et al. 1996; Penesyan et al. 2009). The group of non-pigmented species has highly similar 16S rRNA gene sequences (Ivanova et al. 2004) and is rarely inhibitory against other microorganisms, although, *Pseudoalteromonas haloplanktis* strain INH produces iso-valeric acid and 2-methylbutyric acid showing a broad spectrum of bacterial inhibition (Hayashida-Soiza et al. 2008).

Phylogeny and differentiation of bacterial species rely heavily on 16S rRNA gene similarity (Stackebrandt et al. 2002); however, 16S rRNA gene similarity does not provide sufficient differentiation below species level (Fox et al. 1992). Comparison of secondary metabolite production has supported species delineation within the actinomycete genus *Salinispora*, where strains with high 16S rRNA similarity were shown to belong to distinct species each with different specific metabolite profiles (Jensen et al. 2007). In mycology, comparison of chemical profiles (e.g., TLC, direct-infusion mass spectrometry, and HPLC with various detectors such as UV/VIS and/or mass spectrometry) of secondary metabolites has been widely used to identify and differentiate filamentous fungi (chemotaxonomy) also at sub-species level (Frisvad et al. 2008), and the chemo-phylogeny correlates well with phylogenetic analysis of sequences of specific housekeeping genes (e.g., chitin synthase, β-tubulin, and calmodulin; Geiser et al. 2007). Since several *Pseudoalteromonas* species produce a range of secondary metabolites, we hypothesized that chemical profiling and specific marker compounds could be indicative of bioactive potential and at the same time be useful in species identification or differentiation (Jensen et al. 2007).

The aim of the present study was to profile the secondary metabolites of these strains to determine if particular compounds serve as markers of strains or species with antibacterial activity and to determine if several strains of each species must be tested to assess the full bioactivity potential. As part of this, we provide accurate identification and phylogeny of these organisms by detailed 16S rRNA gene sequence comparative analysis. Since the bacteria were isolated from different sample types, our collection also allows us to address aspects of *Pseudoalteromonas* ecology such as the possible link between surface or planktonic lifestyle and antibacterial activity.

Materials and Methods

Strain Isolation Approximately 500 marine bacterial strains with antagonist activity against *Vibrio anguillarum* strain 90-11-287 (Skov et al. 1995) were isolated during the Danish Galathea 3 research expedition (Gram et al. 2010). One hundred one of these strains tentatively identified as *Pseudoalteromonas* species were included in the present study. *V. anguillarum* 90-11-287 was used as target strain since the expedition ship was not equipped to handle, e.g., potential human pathogens, and this *Vibrio* strain is in our experience very sensitive to antibacterial compounds from other marine bacteria (Hjelm et al. 2004).

Growth Media and Culture Conditions *Pseudoalteromonas* strains were grown in marine broth (MB) 2216 (Difco, Detroit, MI, USA) and on marine agar (MA) 2216 (Difco, Detroit, MI, USA) prepared in accordance with the manufacturer's instructions. Broth cultures were incubated under stagnant conditions at 25°C. Pigment production was determined by visual inspection of 48-h-old culture broths (MB) and colonies grown on MA for 24 to 48 h.

Antibacterial Activity Instant Ocean (IO) bioassay agar plates were prepared as described by Hjelm et al. (2004). Ten grams per liter agar, 3.3 g/l casamino acids (Difco 223050, Detroit, MI, USA), and 30 g/l Instant Ocean aquatic salts (Instant Ocean® Aquarium systems Inc., Sarrebourg, France) were added to distilled water and autoclaved. Glucose (0.4%) and 10 μl/ml of *V. anguillarum* overnight culture were added to the cooled (44°C) IO and plates poured. The plates were allowed to dry for 15 min, and if used for well diffusion agar assays (WDAA), wells (diameter 6 mm) were punched. The inhibitory activity of live *Pseudoalteromonas* bacterial cells was tested by

spotting 48-h-old MA grown colonies on freshly prepared IO agar plates containing *V. anguillarum*. Plates were incubated at 25°C and inspected for clearing zones in the growth of *V. anguillarum* after 24 h.

Cell-free supernatants were prepared to test for the presence of water-soluble antibacterial compounds secreted to the broth, and ethyl acetate extracts were prepared to test for production of non-polar antibacterial compounds. Each strain was grown in 20 ml of MB for 48 h. A 1.5-ml sample was withdrawn for 0.2 μm filtering, and subsequently the remainder of the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate fraction was transferred to a new vessel, evaporated to dryness, and redissolved in 2×0.5 ml ethyl acetate. The 1.5-ml cell-free sterile-filtered supernatant and the ethyl acetate extracts were stored at −20°C until tested in the WDAA (50 μl sample per well) based on IO agar plates containing *V. anguillarum*. Controls (sterile MB and pure ethyl acetate) did not cause any inhibition zones.

The number of antibacterial *Pseudoalteromonas* strains in surface samples (e.g., algae, driftwood, fish, and sediment samples) was compared to their numbers in water samples by the Fisher's exact test (Fisher 1958). A 2×2 contingency table was used to test the hypothesis that presumed antibacterial strains with stable antibacterial activity were equally likely to be isolated from water samples and surface samples.

16S rRNA Gene Sequence Analyses A detailed phylogenetic analysis was performed on 16S rRNA sequences obtained in a previous study (Gram et al. 2010). For the analysis in this study, we conducted a BLAST (http://blast.ncbi.nlm.nih.gov) search against a compilation of *Pseudoalteromonas* type strain sequences retrieved from GenBank (list of type strains obtained from http://www.bacterio.cict.fr), and sequences of the type strains with a BLAST match in our strain collection were included in 16S rRNA sequence analysis to obtain a robust phylogenetic tree. Sequences from two additional *Pseudoalteromonas* strains were included: The genus type strain *P. haloplanktis* and the bioactive *Pseudoalteromonas tunicata*. *Salinispora arenicola* CNS-205 was used as outgroup. The sequences were aligned by the MAFFT online software (http://www.ebi.ac.uk/Tools/mafft/; Katoh et al. 2002) and curated with the Gblocks software on its least stringent settings (Castresana 2000; Talavera and Castresana 2007). The resulting alignment was processed using the MEGA4 software (Tamura et al. 2007) to create neighbor-joining and minimum evolution trees. PhyML 3.0 was used to generate a maximum likelihood tree (Guindon and Gascuel 2003). Phylogenetic trees were generated under default parameters with 1,000 bootstrap replications for neighbor-joining and minimum evolution trees and 100 bootstrap replications for the maximum likelihood tree. GenBank accession numbers for the *Pseudoalteromonas* strains used in this study are included in Supplementary Table 1.

HPLC-UV/VIS Analysis of Secondary Metabolites The strains were grown in static cultures in 10 ml MB for 3 days at 25°C, and for each species, one strain was cultured in triplicate to establish extraction and growth variation. Cultures were extracted with equal volumes of ethyl acetate, centrifuged, and the ethyl acetate was evaporated under N_2 to dryness. Samples were redissolved in 300 μl acetonitrile–water (1:1 *v*/*v*) and filtered through a 13-mm ID PFTE syringe filter. A subsample of 2 μl was then analyzed by reversed phase HPLC on an Agilent 1100 System equipped with a UV/VIS photo diode array detector (scanning 200–600 nm). Separation was done on a 100 mm×2 mm i.d., 3 μm Gemini C_6-phenyl column (Phenomenex, Torrance, CA, USA), running at 40°C using a binary linear solvent system of water (A) and acetonitrile (B; both buffered with 50 μl/l trifluoroacetic acid) at a flow of 300 μl/min. The gradient profile was t=0 min, 5% B; t=22 min, 70% B; t=24.5, 100% B; t=27 min, 100% B; and t=29 min, B=5%, holding this for 8 min prior to the next injection. The chromatographic profiles were compared, subtracting peaks present in media blank extracts. Samples were analyzed in random order, and six of the first extracts were analyzed several times during the sequence to determine any retention time shifts. Cluster analysis was done on a matrix of detected / non-detected peaks (1/0) using NTSYSpc 2.20q (Exeter Software, Setauket, NY, USA). SAHN clustering was used by unweighted pair-group method (UPGMA) and simple distance measurement. Representative extracts were also analyzed by HPLC-UV/VIS-TOFMS in both positive and negative electrospray (Nielsen and Smedsgaard 2003). Peaks were tentatively identified by UV spectra and accurate mass data by matching in Antibase 2009 (35 930 microbial secondary metabolites; Wiley & Sons, Hoboken, NJ, USA; Nielsen et al. 2006).

Results

Pigmentation and Antibacterial Activity The one hundred one *Pseudoalteromonas* strains were originally isolated for their ability to inhibit *V. anguillarum* (Gram et al. 2010). However, on re-cultivation and re-testing for the ability to inhibit *V. anguillarum* after storage at −80°C for several months, only 51 strains retained inhibitory activity (Table 1). These 51 strains were all inhibitory when tested as live cultures in the "spot test" assay. Twenty of the strains produced water-soluble, diffusible, antibacterial substances as indicated by the ability of cell-free sterile-filtered supernatant to inhibit growth of *V. anguillarum* in the

Table 1 Identity, antibacterial activity, and pigmentation of *Pseudoalteromonas* strains from a global collection

16S rRNA cluster	No. of strains		Related type strain[a]	No. of strains from		No. of strains inhibiting *Vibrio*		Inhibition of *Vibrio* by EtAc extracts
	Pigmented	Non-pigmented		Surface samples	Water samples	Pigmented	Non-pigmented	
I	1	37	*P. agarivorans*	16	22	0	0	0
II	5	0	*P. aurantia*	2	3	5	0	0
III	0	9	*P. prydzensis*	8	1	0	0	0
IV	3	3	*P. phenolica*	3	3	3	2	0
V	4	0	*P. luteoviolacea*	4	0	4	0	4
VI	9	0	*P. rubra*	9	0	9	0	0
VII	13	0	*P. flavipulchra*	13	0	13	0	0
VIII	15	0	*P. ruthenica*	14	1	15	0	15
S1727	0	1	*P. mariniglutinosa*	0	1	0	0	0
S3655	1	0	*P. spongiae*	1	0	0	0	0
Total	51	50		70	31	49	2	19

[a] The type strain which the majority of the strains in the cluster were most closely related to

WDAA. These 20 strains were identified as *Pseudoalteromonas phenolica* (one strain), *Pseudoalteromonas luteoviolacea* (five strains), *Pseudoalteromonas rubra* (nine strains), *Pseudoalteromonas citrea* (one strain), and *Pseudoalteromonas aurantia* (four strains). Ethyl acetate extraction of culture broths resulted in 19 crude extracts which inhibited growth of *V. anguillarum* in the WDAA. Four of these were identified as *P. luteoviolacea* and were the only strains where both cell-free supernatant and crude ethyl acetate extracts inhibited *Vibrio* growth. The remaining 15 inhibitory crude extracts all originated from strains identified as *Pseudoalteromonas ruthenica*. The crude ethyl acetate extracts of 26 strains were intensely colored; however, only some of these extracts inhibited growth of *Vibrio* indicating that the pigments were not universally antibacterial. Cell-free culture supernatants and ethyl acetate crude extracts of strains with no growth inhibition of *V. anguillarum* in the "spot test" assay were also tested but showed no growth inhibition.

Forty-eight of the 51 antibacterial strains were pigmented, while two (S3431 and S3655) of the 50 non-active strains were pigmented (Table 1). Antibacterial activity was significantly more likely to be produced by pigmented strains as determined by Fisher's exact test (two-tailed p value of 0.0000). In total, 70 strains were isolated from surface swabs and 31 from water samples. Of the surface-associated strains, 45 remained active in the spot-assay in comparison to six of the water sample strains. The Fisher's exact test demonstrated a significant relation between surface association and stable antibacterial activity (two-tailed p value of 0.0000).

Pseudoalteromonas strains were isolated on all parts of the global cruise in both tropical and temperate waters (Fig. 1). Our strain collection is not large enough to allow for complete biogeographic analysis, but pigmented strains appeared to be more frequent in coastal areas whereas the non-pigmented strains appeared associated with open waters (Fig. 1).

16S rRNA Gene Sequence Analyses We initially performed a BLAST search querying the 16S rRNA gene sequence of each strain against the GenBank database. The results of this analysis were ambiguous, as some sequences returned more than 40 hits all with identical scores in the BLAST results (data not shown), frequently including the sequences of several different *Pseudoalteromonas* species. Therefore, a BLAST analysis was carried out querying the sequences against a complete set of *Pseudoalteromonas* type strains, and hence, each strain is matched with the best type strain BLAST match (Suppl Table 1).

The 16S rRNA gene sequences were used to cluster the strains by constructing a neighbor-joining tree, and branch support was verified by comparison to minimum evolution and maximum likelihood trees (Fig. 2). Nodes supported by an 80% bootstrap cutoff were collapsed when three or more strains were included in the cluster (Fig. 2). An exception was made for clusters VI and VII, which are shown as separate clusters due to obvious differences in phenotype (pigment, bioactivity, secondary metabolite profile). Ninety-nine of the strains fell into one of eight primary clusters.

Clusters I and III consisted of non-pigmented non-inhibitory strains (Table 1). Cluster I included 38 strains and the type strains of *P. haloplanktis*, *Pseudoalteromonas agarivorans*, *Pseudoalteromonas tetraodonis*, *Pseudoalteromonas paragorgicola*, *Pseudoalteromonas distincta*, *Pseudoalteromonas arctica*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas*

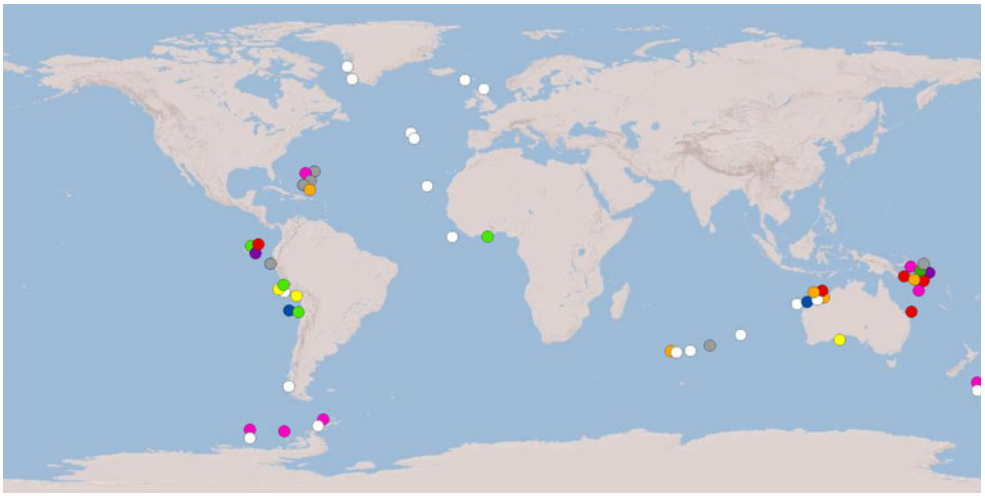

Fig. 1 Isolation sites of 101 *Pseudoalteromonas* strains with each color code representing one of the eight clusters as indicated in Table 1. Each *circle* represents the isolation of one or more strains belonging to the given cluster. *White*=cluster I, *yellow*=cluster II, *gray*=cluster III, *green*=cluster IV, *violet*=cluster V, *red*=cluster VI, *orange*=cluster VII, *pink*=cluster VII, and *blue*=non-clustered strain

elyakovii, *Pseudoalteromonas carrageenovora*, *Pseudoalteromonas marina*, and *Pseudoalteromonas aliena*. Strain S3431—a black-pigmented strain in cluster I—did not show more than 97% similarity even when compared to the full GenBank database which suggests that S3431 could represent a novel *Pseudoalteromonas* species. Despite the low BLAST similarity score, phylogenetic analysis and tree construction placed strain S3431 in the diverse cluster I (the non-collapsed cluster I is shown in Supplementary Figure 1). Cluster III contained no type strains which supported the BLAST analysis where the strains in cluster III were 98% similar to the best type strain match (Supplementary Table 1).

The remaining six of the eight clusters contained pigmented strains. Pale yellow strains clustered with the

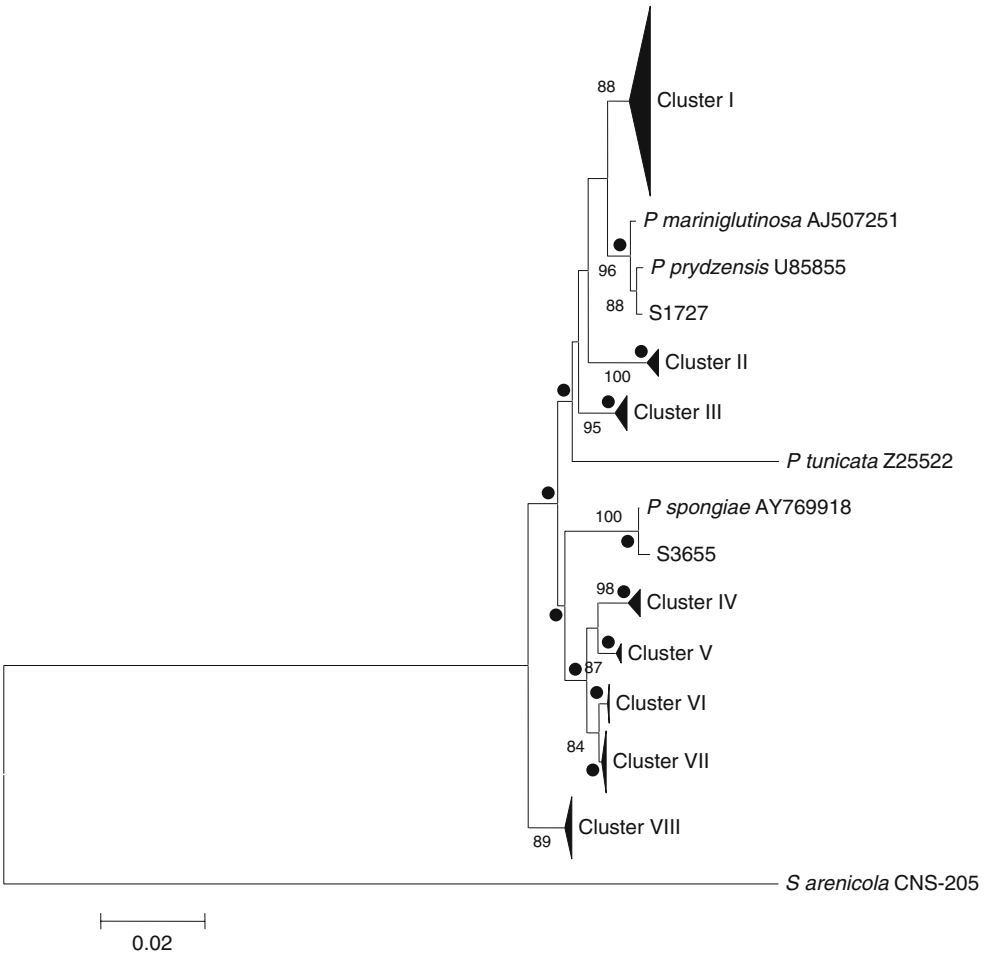

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences of the Galathea 3 *Pseudoalteromonas* strains. Sequences were aligned by MAFFT (default options), and the resulting alignment was used to generate a neighbor-joining tree in the MEGA4 software package (default settings, 1,000 bootstraps). *S. arenicola* CNS-205, GenBank accession number CP000850, GeneID: 5705939 was used as outgroup. Clusters containing two or more non-type strain sequences were collapsed. The *scale bar* represents 0.02 amino acid substitutions per nucleotide position. *Circle* nodes also occurred in minimum evolution and maximum likelihood trees

type strains of *P. citrea* and *P. aurantia* (cluster II) and four intensely purple strains grouped in cluster V with the type strain of *P. luteoviolacea*. Cluster VI contained nine red-pigmented strains and the type strain of *P. rubra*, and cluster VII consisted of 12 intensely yellow strains, one pale yellow strain, and the *Pseudoalteromonas flavipulchra*, *Pseudoalteromonas maricaloris*, and *Pseudoalteromonas piscicida* type strains. Fifteen strains and their nearest BLAST match, *P. ruthenica*, formed cluster VII. These strains all produced a pale brown pigment. Cluster IV contained six strains and the type strain of *P. phenolica*. Four of the strains in this cluster had *P. phenolica* as their best type strain BLAST match; however, strain S1093 had *P. luteoviolacea* as its best match at 98% identity, while *P. rubra* and *P. luteoviolacea* type strains scored identically (97%) as the best matches for S2724. The strains in cluster IV were heterogeneous with respect to pigmentation, some were non-pigmented and others appeared brown.

Profiling of Secondary Metabolites The 101 strains and select type strains were separable in discrete groups by HPLC-UV/VIS (Fig. 3), and the triplicate profiles from an isolate of each species were very reproducible and could be superimposed on each other (data not shown). All of the 38 strains of the 16S rRNA cluster I fell into group A, in which no UV/VIS peaks were unique compared to the media blanks indicating that no secondary metabolites were produced. This large group also included all nine strains from 16S rRNA cluster III and strains of *P. phenolica* and *P. ruthenica* less proficient in secondary metabolite production. A summary of the detected compounds is shown in Table 2, and the producer organisms are shown by 16S rRNA cluster in Table 3.

Based on their production of specific metabolites, the majority of pigmented bacteria formed six main groups not including four *P. rubra* strains (Fig. 3). In the pigmented bacteria, a total of 26 distinct peaks were detected and included in the cluster analysis. We identified indolmycin, violacein, and prodigiosin among the significant peaks based on reference standards. Furthermore, nine peaks could be tentatively identified based on HPLC-UV/VIS-TOFMS results and data in Antibase2009. These nine included two likely novel bromoalterochromides and a brominated indole (Table 2).

Comparing the 16S rRNA gene sequence clusters with the chemical profiling revealed several patterns. Some compounds were exclusively produced by strains belonging to the same cluster whereas other compounds were produced across several strains from different clusters. All strains in cluster II (*P. aurantia*/*P. citrea*) shared production of compound B (retention time, RT 12.31 min) whereas the production of four other compounds F, Q, T, and U (RT 15.47, 16.60, 17.96, and 18.28 min) were scattered in the group. This included a novel bromoalterochromide (compound Q, RT 16.60 min) that was also found in cluster VII (*P. flavipulchra*/*P. piscicida*). Three of the other compounds were identified as quinolines based on distinct UV spectra and accurate mass.

P. luteoviolacea strains (cluster V) shared production of compound D (violacein, RT 14.29) in all four strains and the type strain but were sub-divided by compound A (indolmycin, RT 11.21) produced by two strains and compound Z (pentabromopseudilin, RT 22.65) produced by the two other strains and the type strain. This division is visible in Fig. 4, which shows chromatograms of the four strains in cluster V. Interestingly, also two *P. phenolica* strains produced compound Z.

Cluster VII (*P. flavipulchra* and *P. piscicida*) was chemically very homogeneous. All strains except one produced three bromoalterochromides P, Q, and R (RT 16.49, 16.60, and 17.10) of which Q and R were novel compounds. In contrast to cluster V and compound D, the production of P, Q, and R was not a unique marker for strains of this cluster as P, Q, and R were also detected in one strain from cluster II and one strain from cluster VI.

Thirteen of the 15 strains in cluster VIII, identified as *P. ruthenica*, shared a unique chemical profile and produced the compounds H, K, and O (RT 15.78, 15.98, 16.30) with characteristic UV spectra. None of these matched compounds in Antibase2009 and potentially constitute novel antibacterials. These compounds were not detected among strains from other clusters, yet they were not suitable as a distinct chemical marker for cluster VIII since no secondary metabolites were detected in the remaining two strains in this cluster or the type strain.

The strains in cluster VI were identified as *P. rubra* and were chemically very heterogeneous. Five of nine strains produced the red pigment prodigiosin (compound M, RT 16.00) which was not detected in strains of other clusters. Additionally, a multitude of known and non-identified compounds were detected in one or more strains in the cluster. In total, 16 compounds were detected within the cluster, and 12 of these were unique for this cluster. Only two strains shared an identical production of secondary metabolites, further stressing the chemical diversity among the strains in this cluster.

Discussion

We demonstrate in this study, in agreement with earlier findings (Bowman 2007), that species within the *Pseudoalteromonas* genus produce a range of secondary metabolites, some with antibacterial activity. Several species of the genus are intensely pigmented, and it is

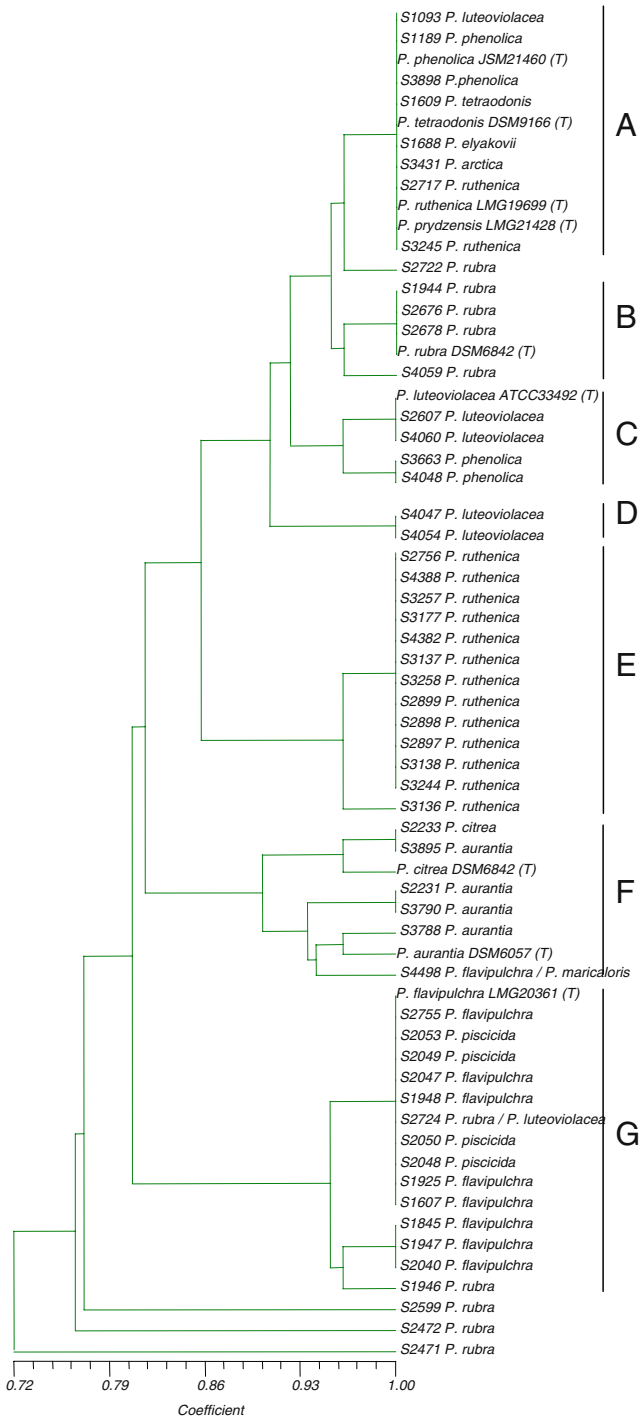

Fig. 3 Dendrogram from cluster analysis of detected peaks from HPLC-UV/VIS detection of compounds in the ethyl acetate extracted broth and biomass. Data were processed in NTSYSpc 2.20q, with SAHN clustering by UPGMA and simple distance measurement

hypothesized that pigmentation co-occur with antibacterial activity (Egan et al. 2002). In our global collection of *Pseudoalteromonas* strains that demonstrated antibacterial activity on initial isolation, strains that were pigmented were significantly more likely to retain antibacterial activity on re-growth than non-pigmented strains. Several intensely colored organic extracts were not inhibitory against *V. anguillarum*, and hence, we do not believe that the pigments, in general, are the cause of the antibacterial activity although, e.g., the purple pigment violacein is a known antibiotic compound (Lichstein and Vandesand 1945).

Table 2 Ethyl acetate extractable secondary metabolites produced by pigmented *Pseudoalteromonas* strains

Compound	MI (Da)[c]	UV-max data	RT (min)	Code
Indolmycin[a]	257		11.21	A
2-Pentyl-4-quinolinol[b]	215		12.31	B
Novel mono-brominated indole	280		13.78	C
Violacein[a]	343		14.29	D
Unidentified	244	212 nm (100%), 250 nm (48%)	15.21	E
2-*n*-Heptyl-(1*H*)-quinolin-4-one[b]	243		15.47	F
Unidentified	NI	228 nm (45%), 308 nm (100%)	15.70	G
Unidentified	386	<200 nm	15.78	H
Unidentified	316	286 nm (100%)	15.80	I
Unidentified	NI	<200 nm	15.81	J
Unidentified	NI	228 nm (45%), 308 nm (100%)	15.98	K
Unidentified	676	<200 nm	15.99	L
Prodigiosin[a]	323		16.00	M
Unidentified	NI	<200 nm	16.26	N
Unidentified	NI	228 nm (45%), 308 nm (100%)	16.30	O
Bromoalterochromide A[b]	843		16.49	P
Novel bromoalterochromide, 2 bromine	921		16.60	Q
Novel bromoalterochromide, 1 bromine	857		17.10	R
Unidentified	333	310 nm (100%)	17.20	S
2-*n*-Nonyl-(1*H*)-quinolin-4-one[b]	271		17.96	T
Nonyl-quinolinone analog[b]	271		18.28	U
Unidentified	315	362 nm (100%)	18.90	V
Unidentified	244	218 nm (100%), 280 nm (82%)	19.42	W
Unidentified	NI	218 nm (100%), 288 nm (82%)	19.78	X
Unidentified	NI	250 nm (100), 280 nm (86)	19.92	Y
Pentabromopseudilin[b]	549		22.65	Z

NI no ionization or MI could not be assigned using ESI^+ and ESI^-

[a] Validated reference standard used for verification

[b] Accurate mass and UV data fit the data from Antibase2009

[c] Mono-isotopic mass

Nearly half of the isolated strains did not retain any antibacterial effect after frozen storage and sub-culturing despite being isolated on the original plates due to antibacterial activity. The observed loss of antibacterial activity may be due to a requirement for factors specific to local seawater, as initial tests for antibacterial activity were carried out using 50% local seawater (Gram et al. 2010). Furthermore, loss of antibacterial activity may be due to repression or inhibition of gene clusters encoding products that are required for secondary metabolite synthesis (e.g., by catabolite repression). A reduction in antibiotic production when the producer organism is grown in excess of a carbon source is a known phenomenon (Sanchez et al. 2010), and suppression of secondary metabolite production by excess concentrations of other substrate components is demonstrated in *Streptomyces* (Doull and Vining 1990). This could suggest that culturing the strains under nutrient limited conditions may reestablish production of antibacterial compounds. Also, during the original sampling and screening procedure, the agar plates may have harbored co-cultured microorganisms which potentially induce antibacterial activity as has been demonstrated by Mearns-Spragg et al. (1998). Hence, it may be possible to re-induce the antibacterial activity if the right conditions can be created.

Several bioactive *Pseudoalteromonas* have been isolated from higher organisms, and it has been hypothesized that antibacterial compounds may play a role in bacterial competition or as protective agents beneficial for the host organism (Holmström and Kjelleberg 1999). We provide statistical evidence that surface-associated presumed antibacterial pseudoalteromonads are significantly more likely to show stable production of antibacterial compounds than

Table 3 Secondary metabolites produced by *Pseudoalteromonas* species clustered by 16S rRNA gene similarity

16S cluster	# strains	Peak at retention time present in *Pseudoalteromonas* strain/organism																									
		None	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
I	38	x																										
P. tetraodonis	DSM9166	x																										
P. prydzensis	LMG21428	x																										
II	1			x				x														x						
	2			x				x														x	x					
	1			x				x											x				x					
	1			x				x											x				x					
P. aurantia	DSM6057			x																	x							
P. citrea	DSM8771			x				x											x									
III	9	x																										
IV	3	x																										
	1																	x	x	x								
	2																											x
P. phenolica	DSM21460	x																										
V	2					x																						x
	2		x			x																						x
P. luteoviolacea	ATCC33492					x																						
VI	1													x														
	1														x			x	x	x							x	
	1				x		x				x														x	x		
	1											x			x						x			x				
	1								x					x	x	x												
	2													x														
	1														x													
	1													x	x													
P. rubra	DSM6842													x														
VII	9																	x	x	x								
	3																	x	x	x							x	
	1			x				x																				
P. flavipulchra	LMG20361																	x	x	x								
VIII	2	x																										
	13									x			x				x											
P. ruthenica	LMG19699	x																										
S1727	1	x																										
S3655	1	x																										

Identification of peak by capital letter in Table 2

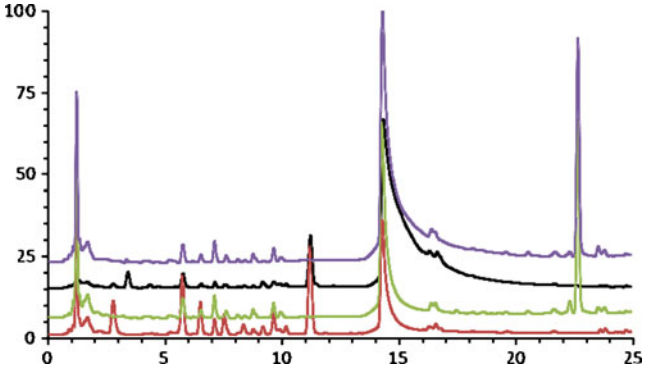

Fig. 4 HPLC-UV/VIS profiles of the ethyl acetate extracted broth and biomass of four *P. luteoviolacea* with the tailing violacein peak at 14–15 min and in two traces pentabromopseudilin at 22-23 min in contrast to indolmycin at 11 min in the two other traces

Pseudoalteromonas species isolated as planktonic cells. This suggests that production of antibacterial compounds may play an important role in the ability of *Pseudoalteromonas* strains to colonize and persist on surfaces submerged in the marine environment, as previously suggested for *P. tunicata* strain D2 (Rao et al. 2005).

The analysis of 16S rRNA gene sequences from our global collection of *Pseudoalteromonas* confirms that phylogenetic analysis results in a number of clusters encompassing predominantly pigmented species or non-pigmented species (Ivanova et al. 2004). Strain S3431 was the single pigmented strain in the so-called non-pigmented clusters. Novel diversity might be represented in cluster III which consisted of strains with 98% or less 16S rRNA gene sequence similarity to *Pseudoalteromonas* type strains and formed a separate cluster in the phylogenetic analysis. However, these strains showed no antibacterial activity and no small molecule metabolites were detected. Such novel diversity could still represent untapped biotechnological potential, producing, e.g., enzymes or peptides with biological activity, as known for other non-pigmented *Pseudoalteromonas* (Hoyoux et al. 2001; Violot et al. 2005).

Chemical profiling of the strains detected an array of secondary metabolites. In addition to complementing our analysis of 16S rRNA gene sequences, it also demonstrated that some compounds (e.g., violacein, prodigiosin) were characteristic of a species and other compounds were produced by several species, and we also detected intra-species clusters of different secondary metabolite profiles. In a broad sense, the clustering based on 16S rRNA gene similarity agreed with the groups resulting from the chemophylogenetic analysis. However, some compounds were produced by organisms of different species that then clustered together using the secondary metabolites as basis. The chemical analysis separated the four isolated *P. luteoviolacea* strains into two distinct sub-groups, showing intra-species chemical diversity. The *P. luteoviolacea* strains produced violacein and pentabromopseudilin which are active against gram-positive and gram-negative bacteria and the anti-staphylococcal agent indolmycin (Hornemann et al. 1971; Hurdle et al. 2004). Violacein and pentabromopseudilin have previously been detected in *P. luteoviolacea* (Gauthier 1976a; Laatsch and Pudleiner 1989), but to our knowledge, this is the first report of *Pseudoalteromonas* strains producing indolmycin (Månsson et al. 2010).

Within some species, all strains were consistently antibacterial. However, in others, such activity did not appear to be a consistent trait of the species. For instance, strains of the 16S cluster VI (*P. phenolica*) were heterogeneous in their ability to inhibit *Vibrio* in our assays, while all but one strain in the homogeneous cluster VII had identical metabolite profiles and all were inhibitory. Even more obvious was the heterogeneous chemical profiles within the *P. rubra* strains. All except one strain shared a chemical marker prodigiosin and/or RT 15.99 min but had major variations in 14 other compounds. This may in part be due to loss of ability to produce a compound. For instance, strain S2471 over time lost ability to produce the brominated indole (RT 13.78 min). Also, we note that the type strain DSM 6842 (ATCC 29570) did not in our culture produce prodigiosin which has been observed previously (Gauthier 1976b; Gauthier and Flatau 1976). The consistent bromoalterochromide production in the two species *P. piscicida* and *P. flavipulchra*/*maricaloris* (cluster VII) was expected (Speitling et al. 2007) and supported the high DNA sequence similarity between the two. This emphasizes the need to isolate and screen multiple strains from each species when bioprospecting within the genus *Pseudoalteromonas*, as even the homogeneous cluster VII contains one strain with a metabolite profile that does not share a single compound with the other strains in this cluster.

Several of the 26 detected peaks were known substances, with a majority known as antibacterials. These included violacein (Lichstein and Vandesand 1945), two bromopseudilins (Lovell 1966), two indolmycins (Werner 1980), four quinolines (Wratten et al. 1977), and prodigiosin (Kalesperis et al. 1975). Due to its very low aqueous solubility, violacein probably protects against predation rather than acts as a true antibiotic, and it has been shown to induce cell death in grazing organisms (Matz et al. 2008). Such compounds would be very beneficial for protection of a biofilm, which is likely how surface-associated *Pseudoalteromonas* would grow. The 14 compounds that could not be identified were mainly not identified due to poor ionization in ESI^+ and ESI^- and/or several plausible candidates in Antibase2009. However, for chemotaxonomic studies, identity of the compounds is not necessary as long as they can be unambiguously identified between samples (Frisvad et al. 2008).

Within cluster VIII (*P. ruthenica*) and cluster II (*aurantia*/*citrea*), we found examples where strains with highly similar

16S rRNA gene sequences (>99%) and with identical chemotaxonomy originated from geographically distinct locations. This latter observation is in agreement with studies on *Salinispora* biogeography and secondary metabolite production in which the authors show how strains of the marine bacterium *S. arenicola* isolated from worldwide locations are highly related and produce identical patterns of secondary metabolites (Jensen and Mafnas 2006; Jensen et al. 2007). In contrast, the *P. luteoviolacea* and *P. rubra* strains showed both local and global variations in their secondary metabolite profile, which one might speculate is due to adaptation to local specific niches.

In conclusion, we believe sampling from specific niches, e.g., biofilms on surfaces, to be of importance in discovery of novel secondary metabolites from the genus *Pseudoalteromonas*. While differences in metabolite patterns among species encourage isolation and screening of novel diversity, bioprospecting known *Pseudoalteromonas* species should not be ruled out. Investigation of multiple strains of one *Pseudoalteromonas* species can yield novel compounds due to intra-species variations within secondary metabolite profiles.

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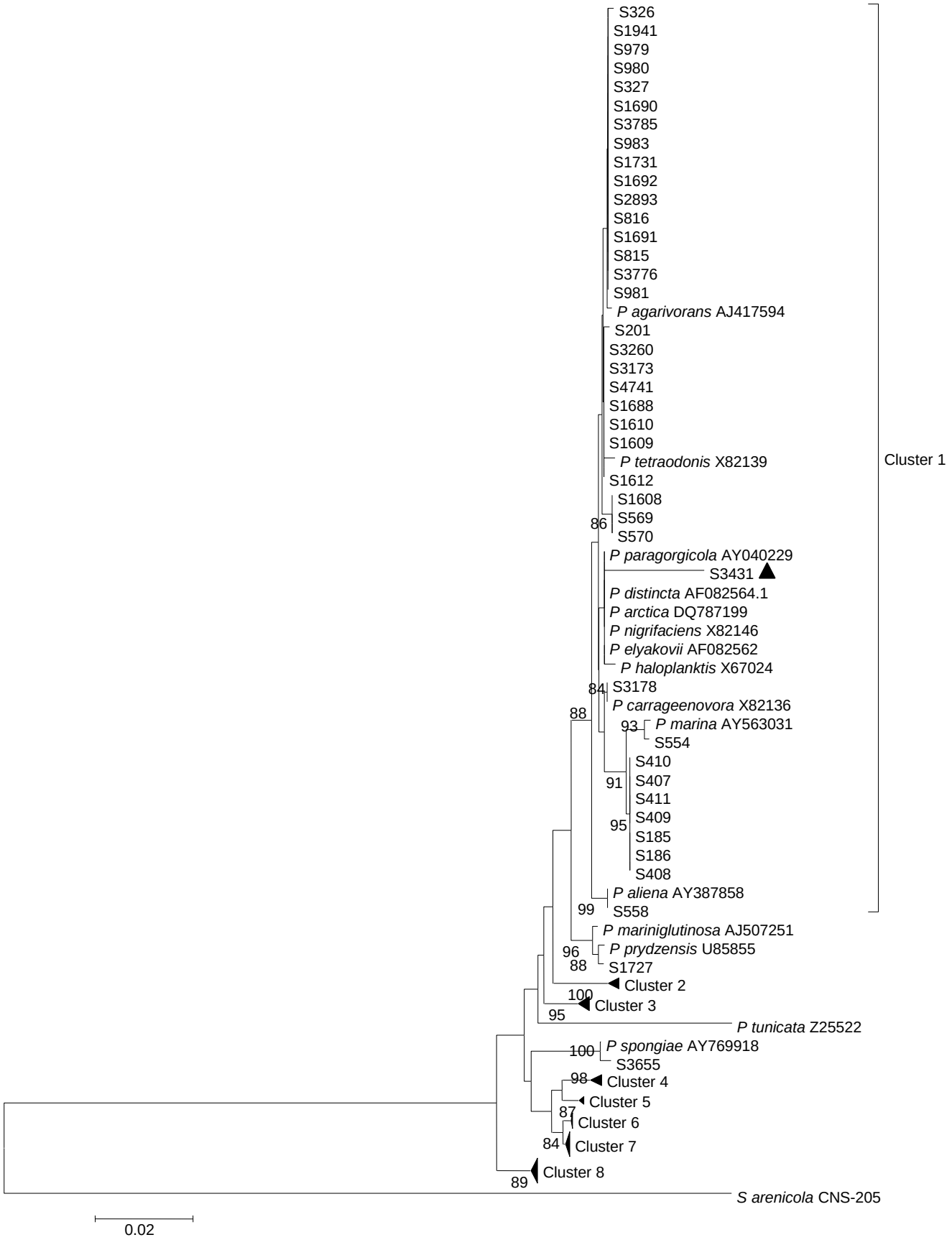
Supplementary material

Figure and table legends

Supplementary Figure 1: The phylogenetic tree also shown in figure 2, here with cluster I not collapsed to provide an overview of the large number of strains contained therein. The filled black triangle marks strain S3431, which in the BLAST analysis showed a low (97%) level of similarity to *Pseudoalteromonas* type strains.

Supplementary Table 1: Origin and anti-bacterial activity of *Pseudoalteromonas* strains included in this study. The cluster designation refers to figure 1. Sequence identity was determined using the BLAST algorithm. Where two hits are listed they scored equally in BLAST analysis.

Supplementary Figure 1: The phylogenetic tree also shown in figure 2, here with cluster I not collapsed to provide an overview of the large number of strains contained therein. The filled black triangle marks strain S3431, which in the BLAST analysis showed a low (97%) level of similarity to *Pseudoalteromonas* type strains.



Supplementary Table 1: Origin and anti-bacterial activity of *Psedoalteromonas* strains included in this study. The cluster designation refers to figure 1. Sequence identity was determined using the BLAST algorithm. Where two hits are listed they scored equally in the BLAST analysis.

Strain	GenBank acc. #	Latitude +=N	Longitude +=E	Water or surface	BLAST type strain match	% BLAST ident.	Cluster 16S	Cluster Chem.	Pigment	Inhibition of *V. anguillarum* Spot-assay	Sup	EtAc ex
S185	FJ457121	62.03815	-9.99592	W	*P. arctica*	98	1	A	-	-	-	-
S186	FJ457122	62.03815	-9.99592	W	*P. distinct*	98	1	A	-	-	-	-
S187	FJ457123	62.03815	-9.99592	W	*P. prydzensis*	97	3	A	-	-	-	-
S201	FJ457124	62.03815	-9.99592	W	*P. agarivorans* *P. distincta*	99	1	A	-	-	-	-
S326	FJ457125	62.2603	-51.6539	S	*P. agarivorans*	99	1	A	-	-	-	-
S327	FJ457126	62.2603	-51.6539	S	*P. agarivorans*	99	1	A	-	-	-	-
S407	FJ457127	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S408	FJ457128	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S409	FJ457129	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S410	FJ457130	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S411	FJ457131	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S554	FJ457133	42.6047	-29.9597	S	*P. marina*	99	1	A	-	-	-	-
S558	FJ457134	42.6047	-29.9597	S	*P. aliena*	99	1	A	-	-	-	-
S569	FJ457136	40.67227	-28.8374	W	*P. tetraodonis*	99	1	A	-	-	-	-
S570	FJ457137	40.67227	-28.8374	W	*P. tetraodonis*	99	1	A	-	-	-	-
S815	FJ457139	23.07925	-24.0523	W	*P. agarivorans*	99	1	A	-	-	-	-
S816	FJ457140	23.07925	-24.0523	W	*P. agarivorans*	99	1	A	-	-	-	-
S979	FJ457141	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S980	FJ457142	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S981	FJ457143	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S983	FJ457144	4.568133	-14.7097	W	*P. agarivorans*	99	1	A	-	-	-	-
S1093	FJ457145	4.570467	-1.72975	W	*P. luteoviolacea*	98	4	A	-	+	+	-
S1189	FJ457146	4.570467	-1.72975	W	*P. phenolica*	98	4	A	Brown	+	+	-

S1607	FJ457149	-38.407	66.3738	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1608	FJ457150	-38.407	66.3738	S	*P. agarivorans*	99	1	A	-	-	-	-
S1609	FJ457151	-38.407	66.3738	S	*P. tetraodonis*	100	1	A	-	-	-	-
S1610	FJ457152	-38.407	66.3738	S	*P. agarivorans* *P. paragorgicola*	99	1	A	-	-	-	-
S1612	FJ457153	-37.2168	72.7091	S	*P. agarivorans* *P. paragorgicola*	99	1	A	-	-	-	-
S1649	FJ457154	-35.3413	79.9641	S	*P. prydzensis*	98	3	A	-	-	-	-
S1650	FJ457155	-35.3413	79.9641	S	*P. prydzensis*	98	3	A	-	-	-	-
S1688	FJ457157	-31.4061	91.17758	W	*P. elyakovii*	99	1	A	-	-	-	-
S1690	FJ457158	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1691	FJ457159	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1692	FJ457160	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1727	FJ457161	-19.7461	114.8573	W	*P. mariniglutinosa*	99	-	A	-	-	-	-
S1731	FJ457162	-19.7461	114.8573	W	*P. agarivorans*	99	1	A	-	-	-	-
S1845	FJ457163	-17.7746	121.8656	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1925	FJ457165	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1941	FJ457166	-17.2706	121.1293	W	*P. agarivorans*	99	1	A	-	-	-	-
S1944	FJ457168	-17.0038	120.7788	S	*P. rubra*	99	6	B	Red	+	+	-
S1946	FJ457169	-17.0038	120.7788	S	*P. rubra*	99	6	G	Red	+	+	-
S1947	FJ457170	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1948	FJ457171	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2040	FJ457173	-16.0604	119.3541	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2047	FJ457174	-16.0604	119.3541	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2048	FJ457175	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2049	FJ457176	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2050	FJ457177	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2053	FJ457179	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2231	FJ457180	-33.3267	127.6573	S	*P. aurantia* *P. citrea*	99	2	F	Pale yellow	+	+	-
S2233	FJ457181	-33.3267	127.6573	S	*P. citrea*	99	2	F	Pale yellow	+	+	-
S2471	FJ457184	-22.9636	153.9486	S	*P. rubra*	99	6	-	Red	+	+	-

S2472	FJ457185	-22.9636	153.9486	S	*P. rubra*	99	6	-	Red	+	+	-
S2599	FJ457186	-10.3454	157.7956	S	*P. rubra*	99	6	-	Red	+	+	-
S2607	FJ457187	-10.3454	157.7956	S	*P. luteoviolacea*	99	5	C	Purple	+	+	+
S2676	FJ457188	-8.0692	155.8781	S	*P. rubra*	99	6	B	Red	+	+	-
S2678	FJ457189	-8.0692	155.8781	S	*P. rubra*	99	6	B	Red	+	+	-
S2717	FJ457190	-7.8244	156.0689	W	*P. ruthenica*	99	8	A	Brown	+	-	+
S2721	FJ457191	-8.1005	156.8451	S	*P. prydzensis*	97	3	A	-	-	-	-
S2722	FJ457192	-8.1005	156.8451	S	*P. rubra*	99	6	-	Red	+	+	-
S2724	FJ457193	-8.1005	156.8451	S	*P. rubra* *P. luteoviolacea*	97	4	G	Brown	+	-	-
S2755	FJ457196	-9.108	156.8595	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2756	FJ457197	-15.2329	156.665	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2893	FJ457198	-48.915	178.1056	S	*P. agarivorans*	99	1	A	-	-	-	-
S2897	FJ457199	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2898	FJ457200	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2899	FJ457201	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3136	FJ457202	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3137	FJ457203	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3138	FJ457204	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3173	FJ457205	-67.4876	-89.1285	S	*P. tetraodonis*	98	1	A	-	-	-	-
S3177	FJ457206	-67.4876	-89.1285	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3178	FJ457207	-67.4876	-89.1285	S	*P. carrageenovora*	99	1	A	-	-	-	-
S3244	FJ457208	-67.4876	-89.1285	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3245	FJ457209	-67.4876	-89.1285	S	*P. ruthenica*	99	8	A	Brown	+	-	+
S3257	FJ457210	-64.0947	-62.8228	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3258	FJ457211	-64.0947	-62.8228	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3260	FJ457212	-64.0947	-62.8228	S	*P. agarivorans*	99	1	A	-	-	-	-
S3431	FJ457214	-50.4498	-74.8912	S	*P. arctica*	97	1	A	Black	-	-	-
S3655	FJ457216	-20.0568	-70.755	S	*P. spongiae*	99	-	A	Orange	-	-	-
S3663	FJ457217	-20.0568	-70.755	S	*P. phenolica*	97	4	C	-	+	-	-
S3776	FJ457222	-14.2295	-76.6073	S	*P. agarivorans*	99	1	A	-	-	-	-
S3785	FJ457223	-14.2295	-76.6073	S	*P. agarivorans*	99	1	A	-	-	-	-

S3788	FJ457224	-17.0857	-72.419	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3790	FJ457225	-17.0857	-72.419	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3895	FJ457226	-14.1631	-77.4286	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3898	FJ457228	-13.818	-76.7648	W	*P. phenolica*	97	4	A	-	-	-	-
S3944	FJ457229	-5.3492	-81.4284	S	*P. mariniglutinosa*	98	3	A	-	-	-	-
S4047	FJ457230	2.9817	-86.6892	S	*P. luteoviolacea*	98	5	D	Purple	+	+	+
S4048	FJ457231	2.9817	-86.6892	S	*P. phenolica*	98	4	C	Brown	+	-	-
S4054	FJ457234	2.9817	-86.6892	S	*P. luteoviolacea*	99	5	D	Purple	+	+	+
S4059	FJ457237	2.9817	-86.6892	S	*P. rubra*	99	6	B	Red	+	+	-
S4060	FJ457238	2.9817	-86.6892	S	*P. luteoviolacea*	98	5	C	Purple	+	+	+
S4382	FJ457239	26.5042	-66.9964	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S4388	FJ457240	26.5042	-66.9964	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S4389	FJ457241	26.5042	-66.9964	S	*P. mariniglutinosa*	97	3	A	-	-	-	-
S4488	FJ457243	24.9963	-67.0246	S	*P. prydzensis*	97	3	A	-	-	-	-
S4491	FJ457244	24.9963	-67.0246	S	*P. prydzensis*	97	3	A	-	-	-	-
S4492	FJ457245	24.9963	-67.0246	S	*P. mariniglutinosa*	98	3	A	-	-	-	-
S4498	FJ457247	24.9963	-67.0246	S	*P. flavipulchra* *P. maricaloris*	99	7	F	-	+	-	-
S4741	FJ457248	58.8041	-3.0564	S	*P. elyakovii*	99	1	A	-	-	-	-

Paper 4

"Antibacterial Compounds from Marine *Vibrionaceae* Isolated on a Global Expedition"

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Article

Antibacterial Compounds from Marine *Vibrionaceae* Isolated on a Global Expedition

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Abstract: On a global research expedition, over 500 bacterial strains inhibitory towards pathogenic bacteria were isolated. Three hundred of the antibacterial strains were assigned to the *Vibrionaceae* family. The purpose of the present study was to investigate the phylogeny and bioactivity of five *Vibrionaceae* strains with pronounced antibacterial activity. These were identified as *Vibrio coralliilyticus* (two strains), *V. neptunius* (two strains), and *Photobacterium halotolerans* (one strain) on the basis of housekeeping gene sequences. The two related *V. coralliilyticus* and *V. neptunius* strains were isolated from distant oceanic regions. Chemotyping by LC-UV/MS underlined genetic relationships by showing highly similar metabolite profiles for each of the two *V. coralliilyticus* and *V. neptunius* strains, respectively, but a unique profile for *P. halotolerans*. Bioassay-guided fractionation identified two known antibiotics as being responsible for the antibacterial activity; andrimid (from *V. coralliilyticus*) and holomycin (from *P. halotolerans*). Despite the isolation of already known antibiotics, our findings show that marine *Vibrionaceae* are a resource of antibacterial compounds and may have potential for future natural product discovery.

Keywords: *Vibrio coralliilyticus*; *Vibrio neptunius*; *Photobacterium halotolerans*; chemotyping; andrimid; holomycin

1. Introduction

Bioactive secondary metabolites are believed to play a key role in microbial interactions by mediating antagonistic activity and intercellular communication [1]. In addition, many microbial natural products have biotechnological potential as antibiotics, biosurfactants, antifungal, or anticancer agents [2]. Sequences of microbial genomes revealed that only a small fraction of the natural product diversity is known, highlighting the potential for finding novel bioactive compounds in environmental microorganisms [3]. The need for novel antimicrobials to combat increasing antibiotic resistances in pathogenic bacteria has stimulated the exploration of other than the traditional sources, such as terrestrial actinomycetes or fungi [4].

The marine environment harbors bacteria with antagonistic traits [5,6], and marine microorganisms are a potential source of novel antimicrobials [7]. Antagonistic marine bacteria have been isolated from surface [8] and deep waters [9], but the majority originated from biotic surfaces such as sponges [10], zooplankton and macroalgae [8,11], corals [12], and bryozoans [13]. Bioactive bacterial strains predominantly belong to *Pseudoalteromonas* spp. [14], the *Roseobacter* clade [15], and *Actinobacteria* [16]. A number of marine-derived antimicrobials have been characterized in greater detail, including halogenated [17] and sulfuric [18] compounds, depsipeptides [19] and lipopeptides [20], glycolipids [21], as well as high molecular weight structures such as amino acid oxidases [22].

Also the *Vibrionaceae* family, Gram-negative *Gammaproteobacteria* ubiquitous in marine and brackish environments [23], harbors strains with antagonistic activity [8]. The family comprises eight genera, with *Vibrio* and *Photobacterium* constituting the majority of species. To date, *Vibrionaceae* have primarily been investigated due to their pathogenic potential to humans and aquatic animals, but they also occur in commensal or symbiotic associations with eukaryotic organisms [23]. While the abundance of *Vibrionaceae* in nutrient-rich microenvironments such as chitinous zooplankton is potentially related to a superior nutrient utilization based on their metabolic versatility [24], antagonism of competing bacteria through production of antimicrobial compounds may also contribute to a selective advantage. Antimicrobials from *Vibrio* spp. can reduce the number of other microbial community members and influence microscale variations in competing bacterial populations [6]. Antibacterial activities have been described from *V. alginolyticus* [25], *V. parahaemolyticus* [26], *V. anguillarum* [27], and several unidentified *Vibrio* spp. [28,29]. However, the nature and frequency of antagonism among vibrios is still largely unknown, and only a few antibiotic *Vibrio* compounds have been structure elucidated to date [30,31].

The present study describes the analysis of bioactive *Vibrionaceae* strains collected during a global marine expedition [8]. The purpose was to (i) provide phylogenetic and chemical analyses of the strains with strongest antibacterial activity; (ii) characterize their bioactivity depending on culture conditions; and (iii) isolate and elucidate the structure of bioactive metabolites. We report the

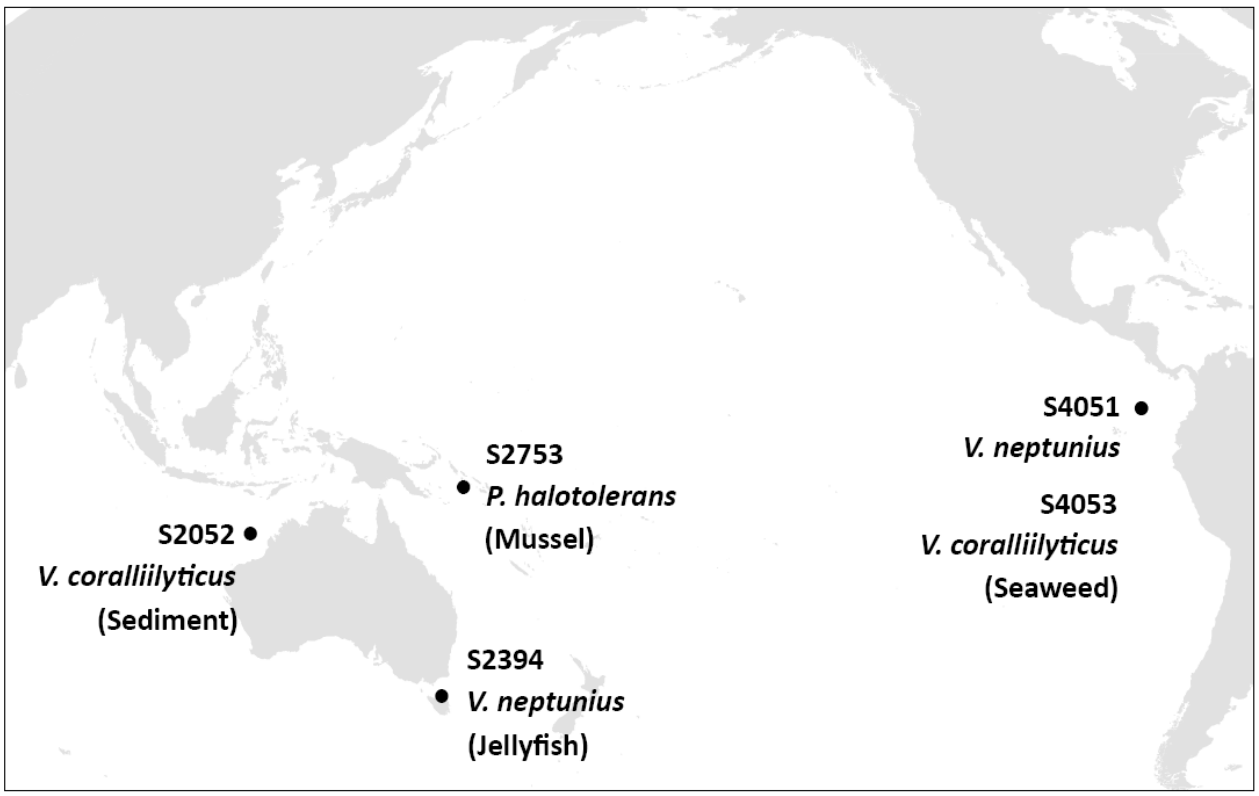
identification of five *Vibrionaceae* strains with pronounced antibacterial activity, the use of chemotyping to support genetic identification, and the structures of two antibacterial compounds.

2. Results and Discussion

2.1. Selection of Strains with Pronounced Antibacterial Activity

Three hundred and one *Vibrionaceae* strains were isolated during a global marine expedition (http://www.galathea3.dk/uk) based on their ability to antagonize the fish pathogen *Vibrio anguillarum* strain 90-11-287 [8]. After being stored at −80 ℃ for between six and 12 months, all strains were retested for antibacterial activity against *V. anguillarum* strain 90-11-287 and the human pathogen *Staphylococcus aureus* strain 8325 by spotting colony mass on pathogen-seeded agar [8]. Activity was assessed by the formation of clearing zones around spotted colony mass. From 301 strains, only 138 retained antibacterial activity, being a small fraction compared to other antagonistic marine bacteria [32,33]. One hundred strains causing pronounced inhibition (diameter of clearing zones larger than 10 mm) were retested using the same set-up, resulting in a subselection of 39 strains with reproducible strong antibacterial activity when spotted on pathogen-seeded agar. This subselection was inoculated in liquid cultures and extracted with ethyl acetate to determine if antibacterial compounds were extractable with organic solvent. Activity was seen in ethyl acetate extracts from five strains, which were selected for further analyses. The five bioactive strains originated from different surface samples collected in distant oceanic regions (Figure 1).

Figure 1. Site of isolation, source, and species identification of five bioactive marine *Vibrionaceae*. Strains were identified to the species level by sequence analysis of several housekeeping genes (see below).



2.2. Phylogenetic Identification and Chemotyping of Strains

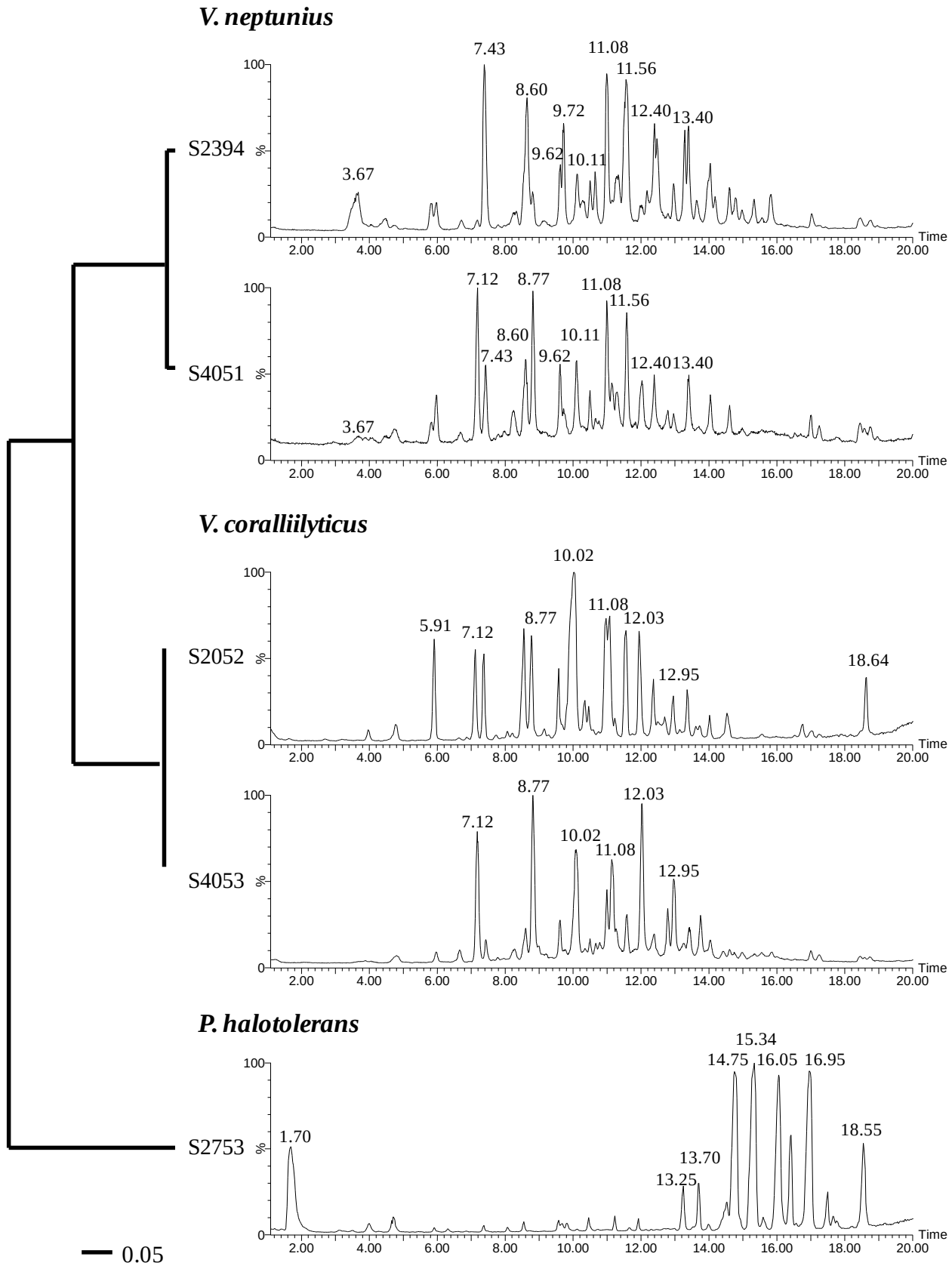
All strains investigated in the present study had previously been assigned to the *Vibrionaceae* family based on 16S rRNA gene similarities [8]. However, the 16S rRNA gene is highly conserved among the *Vibrionaceae* and is not well suited for identification to the species level [34]. Therefore, additional sequence analyses of three housekeeping genes (*recA*, *rpoA*, and *toxR*) were performed. These genes encode constitutively expressed proteins and are suitable for phylogenetic studies of *Vibrionaceae* [34,35]. On the basis of *recA* and *rpoA* sequence similarities, strains S2052 and S4053 were identified as *Vibrio coralliilyticus*, S2394 and S4051 as *Vibrio neptunius*, and S2753 as *Photobacterium halotolerans* (Figure 1). The *toxR* gene was less suited for general species identification due to its high variability even in closely related vibrios, as well as comparatively few *toxR* sequence data available in public gene libraries [36]. However, multiple alignments and neighbor-joining analyses of *toxR* sequences provided the best phylogenetic resolution for determining the relationship between the five strains (Figure 2). The usefulness of *toxR* for species discrimination was consistent with previous reports [35]. LC-UV/MS metabolite profiles underlined the close relationship between *V. coralliilyticus* S2052/4053 and *V. neptunius* S2394/4051, respectively. The evolutionary distance of *P. halotolerans* S2753 to the other strains was reflected by a unique metabolite profile (Figure 2). All five strains were consistent in their metabolite production in separate cultivations over a one-year interval.

Several metabolites were produced by all *V. coralliilyticus* and *V. neptunius* strains, for instance those related to the peaks at retention times Rt = 11.08 and 12.03 min (Figure 2). Although they are different species, *V. coralliilyticus* and *V. neptunius* are closely related vibrios with only 2–3% sequence variation in the *recA* and *rpoA* genes (data not shown), signifying why biosynthetic pathways are shared between the species. Based on their molecular formulas, UV, and MS characteristics [37], most of the metabolites produced by both species were assigned as smaller peptides (*m/z* 300–500), a class of molecules commonly produced by marine culturable bacteria [38,39]. Despite the presence of shared metabolites between *V. coralliilyticus* and *V. neptunius*, clearly distinguishable peaks were seen as well. For instance, the major peak at retention time Rt = 10.02 min (MW 479 Da) was only seen in the two *V. coralliilyticus*, and the peak at Rt = 10.11 min (MW 493 Da) only in the two *V. neptunius* strains.

The metabolites produced by *P. halotolerans* S2753 comprised a series of larger peptides (*m/z* 500–900) [40]. The large peak at Rt = 1.70 min (MW 213 Da) displayed a unique UV spectrum characteristic of that of a highly conjugated system. However, this peak could not be ascribed to any known compound or compound class based on LC-UV/MS data alone.

Several metabolites (Rt = 4.70, 7.41, 8.60, 9.60, and 10.50 min) were found in all five strains and assigned as poly-β-hydroxybutyric acid polymers (PHB) of varying lengths (repeating unit n = 86 Da). This was verified by NMR for some of the compounds (data not shown). PHB are common bacterial storage compounds accumulated when growing on an excess carbon source [41].

Figure 2. Phylogenetic and chemical relationship between five bioactive *Vibrionaceae* based on neighbor-joining analyses of aligned *toxR* gene sequences and LC-MS Total Ion Chromatograms (TIC). The scale bar relates to the number of base substitutions in *toxR* gene sequences (as displayed by branch lengths in the phylogenetic tree).



Chemotyping of prokaryotes has mostly been restricted to analyses of fatty acids and sugars [42], but we show that also the profiling of small molecules can be used for species discrimination. This highlights the usefulness of metabolomics for bacterial classification, adding to recent work of whole-cell laser desorption MALDI-TOF mass spectrometry for characterization of vibrios [43] and secondary metabolite profiling to assess the biosynthetic potential of marine *Pseudoalteromonas* [32]. While our study is limited to the analysis of only three species from the *Vibrionaceae* family, the isolation of two genetically and chemically closely related "strain siblings" from distant oceanic regions indicated that production of certain secondary metabolites is a preserved trait. Similar secondary metabolite profiles were also shown for marine actinomycetal *Salinispora* spp. [44] from distant habitats. Also, all *Ruegeria mobilis* strains from worldwide locations produced the same antibiotic, tropodithietic acid [33].

2.3. Bioassay-Guided Identification of Antibacterial Compounds

V. coralliilyticus (strains S2052 and S4053) and *P. halotolerans* (S2753) inhibited both *V. anguillarum* and *S. aureus*, whereas *V. neptunius* (strains S2394 and S4051) only inhibited *V. anguillarum* (Table 1). Antibacterial activity was highest in aerated cultures and detected after one, three, and five days of incubation. No significant difference in activity was seen between the tested culture media.

Table 1. Inhibition of *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325 by ethyl acetate extracts from five marine *Vibrionaceae*. Antibacterial activity is displayed by the diameter of clearing zones (−: no activity; +: between 0 and 15 mm; ++: between 15 and 30 mm; +++: over 30 mm).

		Inhibition of	
Strain	**Species**	***V. anguillarum***	***S. aureus***
S2052	*V. coralliilyticus*	+++	++
S2394	*V. neptunius*	++	−
S2753	*P. halotolerans*	+++	++
S4051	*V. neptunius*	++	−
S4053	*V. coralliilyticus*	++	+

The finding of bioactivity among marine *Vibrionaceae* underlined marine microorganisms being a source of antimicrobials. To our knowledge, none of the species investigated here have previously been studied with respect to their secondary metabolome including antibacterial compounds.

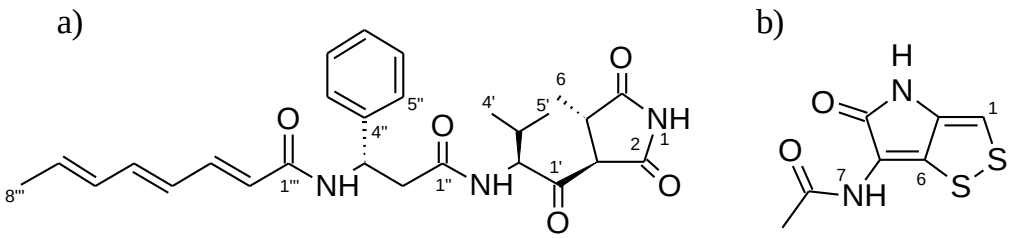
To identify the compounds responsible for the observed activity, large-scale cultivations and fractionations were undertaken for *V. coralliilyticus* S2052 and *P. halotolerans* S2753, representing two distant *Vibrionaceae* species with different metabolite profiles. All fractionation steps were guided by activity testing against *V. anguillarum* strain 90-11-287.

Initial dereplication of S2052 by LC-UV/MS [37] and explorative solid-phase extraction (E-SPE) [45] indicated that andrimid (Rt = 10.02 min; Figure 2) could be responsible for the antibacterial activity. This compound (Figure 3a), a hybrid nonribosomal peptide-polyketide antibiotic, was first described from an insect endosymbiont [46] and later found in other microbial species [47,48] including marine

vibrios [29,31]. Pure andrimid was isolated for NMR analysis, and our data was in accordance with literature data [47]. Andrimid acts as an acetyl-CoA carboxylase inhibitor [49], and we extended its broad antibiotic spectrum [50] by showing inhibition of the bacterial pathogens *Salmonella* Enteritidis, *Bacillus cereus*, *Yersinia enterolitica*, *Yersinia ruckeri*, *Vibrio harveyi*, and *Vibrio vulnificus* (data not shown). Production of andrimid was also confirmed for the other isolated *V. coralliilyticus* strain, S4053. We furthermore speculate whether a recent report of antagonism in the *V. coralliilyticus* type strain [12] was also attributed to this compound. Previous studies have revealed almost identical andrimid gene clusters and a transposase pseudogene in two producer species, suggesting horizontal gene transfer as the most likely explanation behind the cosmopolitanism of the antibiotic [51]. We hypothesize that such transfer is also the reason for its presence in *V. coralliilyticus* S2052 and S4053. Our study is the first linking andrimid production to a specific *Vibrio* species, with production occurring in two strains isolated from very different geographical regions and sources.

The antibacterial compound of *P. halotolerans* S2753 was identified as holomycin (Rt = 1.70 min; Figure 2), a compound belonging to the pyrrothine class of antibiotics acting by interference with RNA synthesis [52]. Our NMR data (Figure 3b) was consistent with previous reports [53]. Holomycin has until now only been found in Gram-positive *Streptomyces* [54,55], and the present study is the first demonstrating production of this antibiotic in a Gram-negative heterotrophic bacterium. While parallel evolution of this trait is possible, horizontal gene transfer is the more likely explanation for its occurrence in both *Vibrionaceae* and actinomycetes. We extended the broad-spectrum activity of holomycin [52] by showing inhibition of the bacterial pathogens *Listeria monocytogenes*, *Serratia marcescens*, *S.* Enteritidis, *B. cereus*, *Y. enterolitica*, *Y. ruckeri*, *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus*, as well as of several marine strains from the *Roseobacter* and *Pseudoalteromonas* groups (data not shown).

Figure 3. Structures of andrimid (**a**) and holomycin (**b**) isolated from marine *Vibrionaceae*.



Neither andrimid nor holomycin were produced by *V. neptunius* S2394 and S4051, and further fractionation and purification is needed to identify the compound(s) responsible for their antibacterial activity. Interestingly, *V. neptunius* S4051 and *V. coralliilyticus* S4053 were isolated from the same seaweed sample, showing that two antagonistic *Vibrio* species whose antibacterial activity is based on different compounds co-occur in the same microenvironment. Moreover, the same sample also contained an antibiotic-producing *Pseudoalteromonas* strain [8].

Two of the antagonistic *Vibrionaceae* species harbor pathogenic strains, with *V. coralliilyticus* being pathogenic to corals [56] and *V. neptunius* being pathogenic to oysters [57]. While we do not know whether *V. coralliilyticus* S2052 has pathogenic potential, the *V. coralliilyticus* type strain has both antagonistic and pathogenic traits [12]. Hence, our results suggest that some vibrios possess a

dual physiology, being antagonistic against other prokaryotes but pathogenic towards higher organisms. Moreover, the production of antibiotics in several species suggests that these compounds may be of ecological importance [1].

This study highlights one of the challenges in natural product discovery. Despite major screening efforts for novel antimicrobials to be used in pharmaceutical, food, and aquaculture industries, only a limited amount of compounds have been discovered in recent years [58]. While the isolation of culturable bacteria remains a promising approach [42] and the secondary metabolome of marine vibrios has not been extensively studied, we only isolated known compounds despite careful dereplication prior to any compound purification. Dereplication is apparently troubled by the high degree of gene transfer between distantly related bacteria such as Gram-positive actinomycetes and Gram-negative *Proteobacteria* [3]. Many compounds in natural product databases such as AntiBase [59] have similar masses (<5 ppm difference), so even the combination of UV/VIS spectra, accurate mass data (<5 ppm), and E-SPE [45] is not sufficiently discriminatory for these organisms. To avoid isolation of redundant chemistry, dereplication by NMR [60] or ultra high-resolution mass spectrometry (<1 ppm) with high isotope accuracy ratios for correct elementary composition determination [61] is imperative to exclude previously isolated compounds.

3. Experimental Section

3.1. Isolation of Bioactive Marine Vibrionaceae

During a global research expedition (http://www.galathea3.dk/uk), marine bacterial strains were isolated from environmental samples and screened for antagonistic activity against a pathogenic *Vibrio anguillarum*, strain 90-11-287. Three hundred and one bioactive strains were identified as *Vibrionaceae* based on 16S rRNA gene similarities [8]. Pure cultures of strains were stored in cryoprotectant solution at −80 °C until being analyzed in the present study.

3.2. Selection of Strains with Pronounced Antibacterial Activity

All 301 strains were retested for antibacterial activity by spotting colony mass on agar seeded with either *V. anguillarum* strain 90-11-287 or *S. aureus* strain 8325. Activity was assessed by the formation of clearing zones around spotted colony mass. Selected active strains were grown both stagnant and aerated (200 rpm) in 30 mL Marine Broth 2216 (Difco 279110) for 3 days at 25 °C in 250 mL glass bottles. Cultures were extracted with an equal volume of HPLC-grade ethyl acetate (EtOAc) for 30 min. The organic phase was transferred to fresh sample vials and evaporated under nitrogen until dryness. Extracts were redissolved in 1 mL of EtOAc and stored at −20 °C until further analysis. EtOAc extracts were tested in a well diffusion agar assay [62] for activity against *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325.

3.3. Phylogenetic Analysis

Genomic DNA was extracted from 1-day cultures using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. PCR for *recA* and *toxR* gene fragments was performed according to [35], and PCR for *rpoA* gene fragments according to [34]. PCR products

were checked by agarose gel electrophoresis and purified using the Wizard PCR Preps DNA Direct Purification System (Promega, Madison, USA) according to the manufacturer's instructions. Obtained nucleotide sequences were edited using Chromas Lite (Technelysium, Australia) and aligned to its closest sequence relative [36]. The phylogenetic relationship between the five isolates was determined by neighbor-joining analyses (1000 bootstrap replicates) of nucleotide and amino acid alignments (translated using EMBOSS Transeq, http://www.ebi.ac.uk/Tools/emboss/transeq/) done in ClustalX. Gene sequences have been deposited at GenBank under the accession numbers HQ452614–452618 (*toxR*), HQ452619–452623 (*recA*), and HQ452624–452628 (*rpoA*).

3.4. Influence of Culture Conditions on Bioactivity

The five strains with strongest antibacterial activity (S2052, S2394, S2753, S4051, and S4053) were grown both stagnant and aerated (200 rpm) at 25 °C in either Marine Broth (MB) or Marine Minimal Medium [63] containing 0.4% glucose and 0.3% casamino acids (MMM). Per strain and culture condition, three bottles were inoculated with 30 mL of medium each, of which each one was sampled after 1, 3, and 5 days of incubation. In addition, strains were grown in 30 mL sea salt solution (Sigma S9883; 40 g L^{-1}) with 0.4% glucose and 0.3% casamino acids for 3 days (200 rpm) at 25 °C. EtOAc extracts were prepared as described above, and tested in a well diffusion agar assay [62] for activity against *V. anguillarum* strain 90-11-287 and *S. aureus* strain 8325.

3.5. Chemotyping

Liquid chromatography-diode array/mass spectrometry (LC-UV/MS) analyses were performed on dried EtOAc extracts redissolved in methanol (MeOH) from all tested culture conditions to visualize the array of produced molecules. In addition, 3-day MMM cultures were extracted and analyzed in biological triplicate. LC-UV/MS was performed on an Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. The separation was done on a Luna II C_{18} column (50 mm × 2 mm, 3 μm) (Phenomenex, Torrance, CA) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (H_2O) to 100% MeCN over 20 min at a flow rate of 300 μL min^{-1}. Both MeCN (HPLC grade) and H_2O were buffered with 20 mM HPLC-grade formic acid (FA).

3.6. Isolation and Structural Elucidation of Antibacterial Compounds

Strains S2052 and S2753 were grown in 20 L sea salt solution (Sigma S9883; 40 g L^{-1}) with 0.4% glucose and 0.3% casamino acids for 3 days (100 rpm) at 25 °C. On day 3, sterile Dianion HP20SS resin (Sigma-Aldrich, St. Louis, MO) was added to the broth (12 g of resin L^{-1}). After 24 h, the resin was filtered off and washed with H_2O (2 × 1 L), followed by extraction with MeCN/H_2O (80/20 v/v; 2 × 1500 mL).

For S2052, all organic extracts were pooled, absorbed onto 90 g Sepra ZT C18 (Phenomenex), and dried before packing into a 100 g SNAP column (Biotage, Uppsala, Sweden) with pure resin (10 g) in the base. Using an Isolera flash purification system (Biotage), the extract was subjected to a crude

fractionation using a MeCN/H_2O gradient (flow rate 30 mL min^{-1}) starting with 10% MeCN (10 min, isocratic), increasing to 100% MeCN (25 min) before washing with 100% MeCN (15 min). Fractions were automatically collected using UV detection (210 and 320 nm). The fraction with antibacterial activity (185 mg) was subjected to further purification on a Luna II C_{18} column (250 × 10 mm, 5 μm) (Phenomenex) using a 45–70% MeCN/H_2O gradient (buffered with 20 mM FA, flow rate 5 mL min^{-1}) over 20 minutes on a Gilson 322 liquid chromatograph with a 215 liquid handler/injector (BioLab, Risskov, Denmark). This yielded 7.6 mg of pure andrimid.

For S2753, the MeCN/H_2O extract from Dianion HP20SS extraction was evaporated until dryness on a rotary evaporator. The extract was redissolved in EtOAc, absorbed onto 5 g Isolute diol (Biotage), and added to a glass column with pure diol (95 g). A total of 12 fractions were collected from the diol column (100 g, 20 × 350 mm) ranging from heptane, dichloromethane, EtOAc to pure MeOH, running under gravity. The fraction with antibacterial activity (172 mg, 100% EtOAc) was further separated on the Isolera flash purification system, on Sepra ZT C18 (10 g SNAP) using a MeCN/H_2O gradient (flow rate 12 mL min^{-1}) starting with 5% MeCN increasing to 30% MeCN (12 min), quickly increasing to 100% MeCN (10 min). Fractions were automatically collected using UV detection (210 and 380 nm). Pure holomycin (4.3 mg) was obtained after final purification on a Luna II C_{18} column (250 × 10 mm, 5 μm) (Phenomenex) using a MeCN/H_2O (buffered with 20 mM FA) gradient from 7–37% MeCN over 17 min.

NMR spectra were recorded on a Bruker Avance 800 MHz spectrometer with a 5 mm TCI Cryoprobe at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules, using standard pulse sequences. The NMR data used for the structural assignment of andrimid and holomycin were acquired in DMSO-d_6 (δ_H 2.49 and δ_C 39.5 ppm).

Optical rotation was measured on a Perkin Elmer Model 341 polarimeter (Perkin Elmer, Waltham, MA) (α_D at 589 nm).

Andrimid: orange-yellow amorphous solid; UV (MeCN/H_2O) λ_{max} 200 (100%), 280 (40%) nm; $[\alpha]_D^{20}$ −62.9 °(*c* 0.24, MeOH); ^{1}H NMR δ_H ppm: 0.75 (3H, d, 6.7 Hz, H-4’), 0.80 (3H, d, 6.7 Hz, H-5’), 1.07 (d, 7.2 Hz, 1H, H-6), 1.78 (3H, d, 6.7 Hz, H-8’’’), 2.30 (m, 1H, H-3’), 2.65 (1H, dd, 14.6, 6.2 Hz, H-2_a’’), 2.77 (1H, dd, 14.6, 8.2 Hz, H-2_b’’), 2.91 (m, 1H, H-4), 3.92 (d, 5.6 Hz, 1H, H-3), 4.62 (dd, 8.4, 5.4 Hz, 1H, H-2’), 5.28 (1H, m, H-3’’), 5.90 (1H, m, H-7’’’), 6.01 (1H, d, 15.2 Hz, H-2’’’), 6.18 (1H, m, H-6’’’), 6.26 (1H, dd, 14.5, 11.4 Hz, H-4’’’), 6.53 (1H, dd, 14.5, 10.0 Hz, H-5’’’), 7.00 (1H, dd, 15.2, 11.4 Hz, H-3’’’), 7.20 (1H, m, H-7’’), 7.29-7.31 (4H, m, H-5’’/H-6’’), 8.11 (1H, d, 8.4 Hz, NH-2’), 8.42 (1H, d, 8.5 Hz, NH-3’’), 11.36 (s, 1H, NH-1); ^{13}C NMR δ_C ppm: 14.5 (C-6), 17.2 (C-4’), 18.3 (C-8’’’), 19.4 (C-5’), 28.1 (C-3’), 39.0 (C-4), 41.9 (C-2’’), 57.8 (C-3), 63.1 (C-2’), 124.2 (C-2’’’), 126.4 (C-5’’), 126.9 (C-7’’), 128.1 (C-4’’’), 128.2 (C-6’’), 131.5 (C-6’’’), 133.4 (C-7’’’), 139.0 (C-5’’’), 139.4 (C-3’’’), 142.9 (C-4’’), 164.3 (C-1’’’), 169.9 (C-1’’), 173.8 (C-2), 180.0 (C-5), 203.9 (C-1’); HRESIMS *m/z* 479.2435 (calcd for $C_{27}H_{33}N_3O_5$, 479.2420).

Holomycin: orange-yellow prisms; UV (MeCN/H_2O) λ_{max} 200 (100%), 280 (40%) nm; ^{1}H NMR δ_H ppm: 2.01 (s, 1H, H-9), 7.04 (s, 1H, H-1), 9.86 (s, 1H, NH-7), 10.69 (s, 1H, NH-3); ^{13}C NMR δ_C ppm: 22.4 (C9), 110.6 (C-1), 115.4 (C-5), 133.7 (C-2), 133.9 (C-6), 167.9 (C-4), 168.8 (C-8); HRESIMS *m/z* 213.9860 (calcd for $C_7H_6N_2O_2S_2$, 213.9871).

4. Conclusions

The present study adds to the knowledge of *Vibrionaceae* bioactivity and physiology by showing a worldwide occurrence of marine strains producing antibacterial compounds. In addition, we underlined that chemotyping can support gene-based species identification and help resolving phylogenetic relationships within a genetically homogenous family such as the *Vibrionaceae*. The discovery of known antibiotics that are also produced by evolutionary distant microbes suggests an involvement of horizontal gene transfer, and indicates that these compounds are fundamental to compete and communicate in the natural habitat. The cosmopolitanism of identical antibiotics has major implications for natural product discovery strategies and stresses the need for careful dereplication in the initial stages of screening. An alternative approach could be the screening for largely untested bioactivities, for instance, interference with quorum sensing or modulation of gene expression.

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Paper 5

"Chitin Stimulates Production of the Antibiotic Andrimid in a *Vibrio coralliilyticus* Strain"

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Chitin stimulates production of the antibiotic andrimid in a *Vibrio coralliilyticus* strain

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Running title – Andrimid production in *V. coralliilyticus*

Keywords: *Vibrio coralliilyticus*, andrimid, chitin, seaweed, biosynthetic temperature optima, secondary metabolism

Summary

Vibrio coralliilyticus is a putative coral pathogen in tropical oceans, but also possesses antagonistic traits. We previously reported antibacterial activity in *Vibrio coralliilyticus* strain S2052 based upon the antibiotic andrimid. The purpose of the present study was to determine whether the antibiotic is produced under conditions mimicking natural habitats of vibrios. *V. coralliilyticus* S2052 synthesized andrimid with both chitin and seaweed extracts as sole nutrient source. In laboratory medium, S2052 produced a range of secondary metabolites, including andrimid. With chitin, the biosynthesis of metabolites other than andrimid was largely abolished, and the yield of the antibiotic was two-fold higher. The metabolic focus on andrimid production with chitin indicates that the antibiotic serves an ecophysiological function. Comparison of S2052 with two related *V. coralliilyticus* strains (LMG20984^T and LMG10953) revealed physiological differences between these close relatives. Despite overall similar secondary metabolomes, LMG20984^T and LMG10953 did not produce andrimid, and their optimum biosynthetic temperature was 30 as compared to 25 °C for S2052. Although the true pathogenic potential of S2052 is unknown, we showed that it contains a zinc metalloprotease gene linked to coral disease. Different physiologies of S2052 and closely related strains indicated that *V. coralliilyticus* subspecies may be adapted to different niches.

(195 words)

Introduction

The *Vibrionaceae* are a diverse family of Gram-negative heterotrophic bacteria commonly found in marine and brackish environments. Vibrios contain several commensal and symbiotic species, but are primarily researched due to their pathogenic potential towards man and marine eukaryotes (Thompson et al., 2004). *Vibrio coralliilyticus* is a model organism for the study of coral disease, being responsible for bleaching and tissue lesions in *Pocillipora damicornis* (Ben-Haim et al., 2003b) and the potential aetiological agent of White Syndrome throughout the Indo-Pacific (Sussman et al., 2008). The physiological and genetic background of its virulence (Sussman et al., 2009; Meron et al., 2009) includes a characteristic temperature-dependent pathogenicity which only occurs above 25 °C (Ben-Haim et al., 2003a). Increasing seawater temperatures due to global warming may therefore facilitate outbreaks of coral disease by *V. coralliilyticus* and other pathogens (Rosenberg and Ben-Haim, 2002). The species is also able to infect crustaceans, rainbow trout (Austin et al., 2005), and bivalve larvae (Ben-Haim et al., 2003a). There is substantial genetic variation between *V. coralliilyticus* strains, indicating the existence of endemic populations in different oceanic regions (Pollock et al., 2010a). Potentially, this could also be reflected in a presence of strains with different niches based on physiological variation, for instance their pathogenicity or metabolic activity.

The *Vibrionaceae* also harbour strains with antibacterial activity (Long and Azam, 2001), and several antagonistic vibrios, including *V. coralliilyticus*, were recently isolated from the marine environment (Gram et al., 2010). *V. coralliilyticus* strain S2052 was isolated from sediment in the tropical Indian Ocean, and antagonized bacterial pathogens by production of the antibiotic andrimid (Wietz et al., 2010). Also the *V. coralliilyticus* type strain has antibacterial activity (Rypien et al., 2010), but it has not been determined whether this was related to the same compound. Andrimid is a hybrid nonribosomal polyketide-peptide antibiotic and effective against a

wide range of bacteria (Singh et al., 1997) by interference with fatty acid synthesis (Freiberg et al., 2004). The antibiotic is also produced by other microbes, including an insect endosymbiont (Fredenhagen et al., 1987), an enterobacterium (Jin et al., 2006), as well as marine pseudomonads (Needham et al., 1994) and vibrios (Oclarit et al., 1994; Long et al., 2005). The cosmopolitan occurrence of andrimid is likely related to horizontal gene transfer (Fischbach, 2009).

Many studies of bacterial physiology are conducted using laboratory substrates that do not reflect conditions in the natural habitat. However, laboratory growth conditions can shift the phenotype towards a metabolic state that is potentially unlike the environmental ecology of a microorganism (Palkova, 2004). The source of carbon (Sanchez et al., 2010) and nutrient availability (Demain et al., 1983) can have a substantial influence on secondary metabolism. An excess of nutrients – typically not encountered in the oligotrophic marine environment – can increase the synthesis of storage compounds, for example polyhydroxybutyrates (Chien et al., 2007), but suppress antibiotic production (Doull and Vining, 1990).

Also the presence of a natural growth substrate, such as chitin, can influence bacterial physiology. Chitin is the most abundant biopolymer in the marine environment, particularly found in the exoskeletons of crustaceans and zooplankton (Gooday, 1990), and profoundly linked to the ecology of vibrios. In the human pathogen *V. cholerae*, chitin influences bacterial ecophysiology at multiple hierarchical levels including chemotaxis, biofilm formation, nutrient cycling, pathogenicity, as well as commensal and symbiotic relationships with higher organisms (Pruzzo et al., 2008). Vibrios are often abundant in chitinous microenvironments (Thompson et al., 2004) due to their ability to utilize chitin as carbon and nitrogen source by secretion of chitinases (Hunt et al., 2008). Vibrios also occur in epiphytic communities of macroalgae, including the brown seaweed *Laminaria* (Laycock, 1974), which is potentially linked to the degradation of algal-derived carbohydrates (Goecke et al., 2010). The utilization of chitinous and algal substrates, possibly

97 combined with the production of antagonistic compounds, could provide vibrios with a selective
98 advantage to colonize biotic surfaces.
99 The purpose of the present study was to determine whether *V. coralliilyticus* S2052
100 produces the antibiotic andrimid under conditions mimicking natural habitats of vibrios. Production
101 of andrimid with seaweed extracts and stimulated biosynthesis with chitin suggested an ecological
102 role of the antibiotic. Furthermore, comparison of antibiosis, secondary metabolism and potential
103 pathogenicity traits (Sussman et al., 2008; 2009) in S2052 and two related strains suggested that *V.*
104 *coralliilyticus* harbours several subspecies with different antagonistic potential and
105 ecophysiological characteristics.
106
107
108 **Results**
109
110 **Andrimid production under natural growth conditions**
111 *V. coralliilyticus* S2052 inhibited pathogenic bacteria by production of the antibiotic
112 andrimid when grown in laboratory medium (Wietz et al., 2010). Here, we investigated growth and
113 andrimid production under natural conditions with chitin, seaweed extracts, or pure algal
114 carbohydrates as sole nutrient source. S2052 grew well in chitin medium, and chitinolytic activity
115 was visually detected as disappearance of the colloidal chitin precipitate. Antibacterial activity and
116 production of andrimid was confirmed by bioassay testing against *V. anguillarum* strain 90-11-287
117 and LC-UV/MS analyses with comparison to an NMR validated standard. With chitin, S2052
118 abolished the production of an array of metabolites produced in laboratory medium (Fig. 1) that had
119 been assigned as polyhydroxybutyrates and smaller peptides by NMR spectroscopy (Wietz et al.,
120 2010).

In seaweed media, the type and concentration of seaweed extract influenced growth characteristics and andrimid production. With *Fucus*, only 50 and 100% extract concentration yielded distinct growth. Bioactivity and considerable production of andrimid was detected with 50, but hardly any with 100% extract concentration. With *Laminaria*, high cell densities were reached at 25, 50, and 100%. Bioactivity and production of andrimid was confirmed for all extract concentrations. We tested whether the observed bioactivities reflected an ability to degrade known components from *Fucus* and *Laminaria* likely present in the crude extracts. Therefore, the polysaccharide fucoidan and its main monomer fucose (mainly present in *Fucus*), the polysaccharide laminarin (mainly present in *Laminaria*), as well as the polysaccharide alginate and the monosaccharide mannitol (present in both), were tested as sole carbon source. Distinct growth was only seen with 1% alginate; however, no andrimid was produced as confirmed by bioactivity testing and LC-UV/MS.

Growth and quantification of andrimid in chitin versus laboratory media

In laboratory medium, there was no lag phase and exponential growth started immediately. Andrimid was detected after 10 hours at an approximate culture density of 5×10^8 cells mL^{-1}. The yield of andrimid (production per cell) peaked after 10 h, reaching approx. 10 pmol per cell. Despite continuing growth between 12 and 24 h, the first decreasing and then stagnant yield illustrated that no additional andrimid was produced (Fig. 2). With chitin, there was a three-hour lag phase before exponential growth started, and the generation time was longer than in laboratory medium. Andrimid production was detected after 18 h and at a slightly lower culture density (10^7 cells mL^{-1}). The yield peaked after 24 h at over 20 pmol per cell, being two-fold higher than with laboratory medium. Production stopped along with an increase in growth between 24 and 30 h. The slightly decreasing yield towards the end of the incubation period indicated that the antibiotic was

145 partially degraded with time. UPLC-UV analyses of ethyl acetate extracts obtained at every
146 sampling point underlined earlier observations (Fig. 1) in a time-dependent manner, showing that
147 over 72 h very few metabolites other than andrimid were produced when grown with chitin.
148
149 **Influence of temperature on metabolite production in S2052 and related strains**
150 We compared the production of andrimid and metabolite profiles of S2052 with two
151 related *V. coralliilyticus* strains, LMG20984^T and LMG10953. Since the pathogenic potential (Ben-
152 Haim et al., 2003a) and intercellular chemistry (Boroujerdi et al., 2009) of *V. coralliilyticus* is
153 temperature-regulated, we tested growth at 15, 20, 25, 30, and 35 °C to see whether temperature
154 also influences secondary metabolite and antibiotic production.
155 All strains grew from 15 to 35 °C, with fast doubling times (estimated 20-30 minutes)
156 above 30 °C. The three strains had almost identical secondary metabolite profiles, but andrimid
157 (peak at 10.01 min) was only produced by S2052. In contrast, another metabolite (peak at 9.87 min)
158 was mainly produced by LMG20984^T and LMG10953 (Fig. 3). S2052 produced andrimid at 15, 20
159 and 25 °C, but only limited amounts at 30 and not at all at 35 °C. We tested whether the lack of
160 production above 30 °C was related to compound degradation over the 3-day incubation period, yet
161 andrimid was neither detected in cultures incubated for only 12 h. Temperature optima for
162 secondary metabolite production differed between the strains. Higher relative abundances of
163 metabolites revealed that S2052 had highest biosynthetic capacity at 25 °C, while only limited
164 amounts were produced at 30 °C. In contrast, LMG20984^T and LMG10953 had the maximum
165 biosynthetic capacity at 30 °C (Fig. 3). These physiological distinctions questioned the
166 identification of S2052 as *V. coralliilyticus*, however, we verified the species affiliation by
167 successful amplification of a *dnaJ* gene fragment using *V. coralliilyticu*s specific primers (Pollock
168 et al., 2010b). The relationship of S2052 to the demonstrated coral pathogens LMG20984^T and

LMG10953 was furthermore underlined by amplification of a zinc metalloprotease gene fragment linked to pathogenicity (Sussman et al., 2008; 2009).

Discussion

The *Vibrionaceae* family comprises symbiotic, commensal, pathogenic (Thompson et al., 2004), as well as antagonistic strains (Long and Azam, 2001; Gram et al., 2010). The relatively widespread production of antibiotics in marine vibrios including *V. coralliilyticus* (Wietz et al., 2010) indicated that antagonistic activity may be of ecological importance. To further test this hypothesis, the present study investigated andrimid production in *V. coralliilyticus* S2052 under conditions mimicking natural habitats of vibrios. Antagonism based upon the utilization of natural substrates could facilitate the colonization of biotic surfaces, and contribute to the association of vibrios with zooplankton (Thompson et al., 2004) and macroalgae (Laycock 1974).

Chitinolytic activity is a core function of the *Vibrionaceae* (Hunt et al., 2008), making it a potential reason for their ubiquitous occurrence (Riemann and Azam, 2002). Chitin controls several genetic and physiological characteristics of vibrios (Pruzzo et al., 2008), and the present study showed that it also influences antagonistic activity. The stimulation of antibiotic production by chitin was potentially related to observations in streptomycetes (Rigali et al., 2008), the paradigm of antibiotic-producing bacteria (Hopwood, 1999). The almost complete shut-down of metabolite production except for andrimid indicated that all available carbon was used for its biosynthesis, suggesting that a competitive phenotype was formed that could be of advantage in the natural habitat. We can, however, only speculate about the ecological role of andrimid. The combination of chitinolytism and antibiosis could provide a selective advantage in the colonization of chitinous microenvironments by using andrimid to antagonize competing bacteria. Andrimid

could also represent a chemical mean to provide grazing resistance. Such a scenario has been shown for the bioactive bacterial compound violacein, which has both antibacterial and antiprotozoan activity (Matz et al., 2004). The link between chitin and andrimid could also play a role in pathogenicity, comparable to the importance of chitinase activity in *V. cholerae* infections (Kirn et al., 2005). Considering the ubiquity of quorum sensing in vibrios including *V. coralliilyticus* (Tait et al., 2010) we hypothesized that andrimid production and/or chitinolytism would be controlled by such mechanisms. Although quorum sensing can be linked to chitinase activity (Chernin et al., 1998; Defoirdt et al., 2010) we did not detect any signalling molecules using two different microbiological AHL monitors (McClean et al., 1997; Cha et al., 1998) (data not shown).

We hypothesized that variations in growth and andrimid production between *Fucus* and *Laminaria* extracts reflected differing abilities of S2052 to utilize species-specific algal compounds. Degradation of fucoidan (Furukawa et al., 1992) and laminarin (Alderkamp et al., 2007) has been shown in vibrios, but S2052 was unable to utilize these as sole nutrient source. In contrast, S2052 could utilize alginate, but no andrimid was produced. In combination with missing antibiosis in undiluted compared to diluted *Fucus* extract, this suggested that biosynthesis of the antibiotic is not a constitutive trait, requires a mixture of algal nutrients, and can be influenced by substrate type or concentration.

The almost identical metabolite profiles of S2052, LMG20984^T and LMG10953, together with the presence of a conserved *dnaJ* gene fragment, confirmed that all three strains belong to *V. coralliilyticus*. However, the production of andrimid in S2052 but its absence in LMG20984^T and LMG10953 showed that even such closely related strains can possess considerable physiological variation. Missing production in LMG20984^T furthermore implied that its antagonism against coral-associated bacteria (Rypien et al., 2010) is caused by a compound that is untraceable in our approach. Nevertheless, the increased antibiosis of LMG20984^T at 25

compared to 30°C was potentially related to the observation in S2052. In combination, these results suggest that antagonistic interactions involving vibrios play a greater role at lower temperatures. Stopping andrimid production above 30 °C was consistent with another andrimid-producing *Vibrio* (Long et al., 2005), and potentially related to the fact that the synthesis of polyketides and nonribosomal peptides can be thermoregulated (Rohde et al., 1998).

The increased biosynthetic capacity of the known pathogens LMG20984^T and LMG10953 at higher temperatures was consistent with a previous report using metabolomics (Boroujerdi et al., 2009), and probably reflected that pathogenicity only occurs above 25 °C (Ben-Haim et al., 2003a). The higher metabolite production of S2052 at lower temperatures implied that its pathogenic potential may differ from LMG20984^T and LMG10953. However, the presence of a zinc metalloprotease gene linked to disease signs in corals (Sussman et al., 2008; 2009) contradicted this hypothesis. It has to be noted, though, that the presence of the gene does not necessarily imply pathogenicity, since coral disease such as White Syndrome is likely multifactorial (Sussman et al., 2008). The observation of two different phenotypes – one pathogenic, non-andrimid producing with a temperature optimum of 30 °C (LMG20984^T and LMG10953) and one andrimid-producing with potential pathogenicity and a temperature optimum of 25 °C (S2052) – possibly reflects genetic variability (Pollock et al., 2010a) and differing enzymatic capacities (Ben-Haim et al., 2003a) among *V. coralliilyticus*. Differing physiologies could even relate to geographically separated, endemic populations within the species (Pollock et al., 2010a). Our results suggest that bacterial taxonomy could benefit from complementation by metabolite analyses (Gevers et al., 2005). The potential of metabolomics for bacterial typing has been exemplified with 12 strains of *E. coli*, showing that all had a different metabolic fingerprint and only shared a core set of compounds (Maharjan and Ferenci, 2005). Also strains from the marine bacterium *Pseudoalteromonas*

luteoviolacea could be grouped into subspecies according to their secondary metabolome (Vynne et al., under revision).

In conclusion, production of andrimid with seaweed extracts and stimulated biosynthesis with chitin implied that antagonistic traits in *V. coralliilyticus* serve an ecological function. Antagonism based upon the utilization of natural substrates could provide a competitive advantage, facilitate the colonization of biotic surfaces, or play a role in pathogenicity. The presence of different phenotypes among *V. coralliilyticus* indicated the existence of several subspecies with different ecophysiogical characteristics. The variations in antibiosis and biosynthetic temperature optima suggested that closely related *V. coralliilyticus* strains may occupy different niches and respond differently to environmental variation.

Experimental procedures

Preparation of natural growth substrates. Colloidal chitin was prepared from practical grade chitin (Sigma P7170) as follows: 10 g chitin were hydrolyzed in 400 mL ice-cold 37% HCl for 20 min and stirred at 37 °C until clear. The solution was poured into 4 L of dH_2O and placed at 4 °C overnight for settlement of chitin. The supernatant was aspirated and chitin resuspended in 2 L of dH_2O. Chitin was collected by centrifugation (4000 *g* for 12 min) and resuspended in 1 L dH_2O. The pH was adjusted to 7 using KOH pellets. The solution was homogenized for 5 min using an Ultra-Turrax (IKA, Staufen, Germany) and autoclaved. The final concentration of colloidal chitin was determined from a dried (70 °C overnight) subsample. Aqueous seaweed extracts were prepared from the brown macroalgae *Fucus vesiculolus* and *Laminaria saccharina* freshly collected from Danish coastal waters. Algae were cut in strips and homogenized in 8 mL sea salt solution

(Sigma S9883, 40 g L^{-1}) per 1 g algae using an Ultra-Turrax (IKA). Extracts were centrifuged (15 min at 2000 *g*) to precipitate larger pieces, filtered through Whatman No 1, and centrifuged again (10 min at 6000 *g*) to remove remaining particles. Extracts were sterilized by filtration (0.2 µm) and stored at 4 °C. The pure algal carbohydrates fucoidan (Sigma F5631; 1% w/v), fucose (Sigma F8150; 10% w/v), laminarin (Sigma L9634; 2% w/v), and mannitol (Sigma M4125; 10% w/v) were readily dissolved in dH_2O. A 1% alginate solution was prepared by slow addition of alginate (Sigma 05550) and sea salts (40 g L^{-1}) to dH_2O to prevent gelling. All solutions were sterilized by filtration (0.2 µm).

Bacterial strains and growth conditions. *V. coralliilyticus* S2052 (Gram et al., 2010) was inoculated in marine minimal medium (MMM) (Östling et al., 1991) containing (i) 0.2% colloidal chitin, (ii) 10, 25 and 50% of seaweed extracts, and (iii) 0.05, 0.2 and 0.4% of algal carbohydrates. In addition, undiluted (100%) seaweed extracts, as well as stock solutions of fucoidan/alginate (1%) and laminarin (2%) amended with sea salts (40 g L^{-1}), were tested as growth substrate. Furthermore, S2052 was inoculated in MMM containing 0.4% glucose and 0.3% casamino acids, representing laboratory medium. Cultures were grown aerated (200 rpm) at 25 °C for 3 (laboratory medium, chitin, 1% alginate and 50/100% seaweed extract) or 7 days (10/25% seaweed extract). Metabolite production in S2052 was compared to two related strains ($LMG20984^T$ and LMG10953). Cultures were grown aerated (200 rpm) in each 30 mL MMM with 0.4% glucose and 0.3% casamino acids at 15, 20, 25, 30, and 35 °C for 3 days. All strains were furthermore screened by PCR for a *dnaJ* gene fragment conserved in *V. coralliilyticus* (Pollock et al., 2010b) and the presence of a *Vibrio* zinc metalloprotease gene (Sussman et al., 2008).

Andrimid detection. All cultures were extracted with an equal amount of HPLC-grade ethyl acetate for 30 min. The organic phase was transferred to fresh sample vials and evaporated under

nitrogen until dryness. The residue was redissolved in 1 mL of ethyl acetate and tested for activity against *V. anguillarum* strain 90-11-287 using a well diffusion agar assay (Hjelm et al., 2004). Blank samples representing all tested culture conditions were analyzed to exclude antibacterial activity by any components of the growth substrates. All active samples were analyzed for the presence of andrimid by liquid chromatography-diode array/mass spectrometry (LC-UV/MS). Extracts were redissolved in methanol and analyzed on an Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. Separation was obtained on a Luna II C_{18} column (50 × 2 mm, 3 μm; Phenomenex, Torrance, CA) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (both buffered with 20 mM formic acid) increasing to 100% MeCN over 20 minutes at a flow rate of 0.3 mL min^{-1}. In LC-UV/MS profiles of seaweed cultures, the bacterial metabolites were camouflaged by a large number of co-extracted algal compounds, but andrimid could be detected by its high-resolution molecular mass using the selective ion-trace (*m/z* 480.25, ESI^+).

Growth kinetics of S2052. S2052 was inoculated at approx. 10^3 cells mL^{-1} in 300 mL MMM with (i) 0.4% glucose and 0.3% casamino acids and (ii) 0.2% colloidal chitin (each in duplicate). Cultures were grown aerated (200 rpm) at 25 °C. Sampling points were set according to growth characteristics in a preliminary test (data not shown). Glucose/casamino acid cultures were sampled after 0, 2, 4, 6, 8, 10, 12, 15, 24, 48 and 72 h, and chitin cultures after 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 48 and 72 h. At each time point, 12 mL of culture were sampled. A serial dilution series was plated on Marine Agar 2216 (Difco 212185) and determination of colony forming units (CFU mL^{-1}) performed after 2 days of incubation at 25 °C.

Andrimid quantification. At each sampling point, the amount of andrimid was quantified directly from sterile-filtered (0.2 µm) culture using ultra-high liquid chromatography-diode array (UPLC-UV) analyses on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with a diode-array detector. Separation was obtained on a Kinetex C_{18} column (150 × 2.1 mm, 2.6 µm; Phenomenex) maintained at 60 ºC using a linear gradient starting from 15% MeCN in water (both buffered with 50 ppm trifluoroacetic acid) increasing to 100% MeCN over 7 minutes at a flow rate of 0.8 mL min^{-1}. Injection volume was 20 µL. Andrimid (Rt = 4.44 min) was detected from the 302 ± 2 nm chromatogram using quantification by external standard calibration with an NMR validated standard of pure andrimid (Wietz et al., 2010). A standard curve from six different andrimid concentrations (0.06, 0.2, 0.3, 0.45, 0.65, and 2.5 mM) resulted in a linear calibration curve with R^2 = 0.9991. In addition, 5 mL culture from each time point were extracted twice with ethyl acetate (with and without 1% formic acid) to investigate the overall production of secondary metabolites over time. The extracts were pooled and evaporated under nitrogen until dryness. The residue was redissolved in 1 mL methanol and filtered (0.45 µm) before subjected to UPLC-UV analyses as described above using a 3 µL injection volume.

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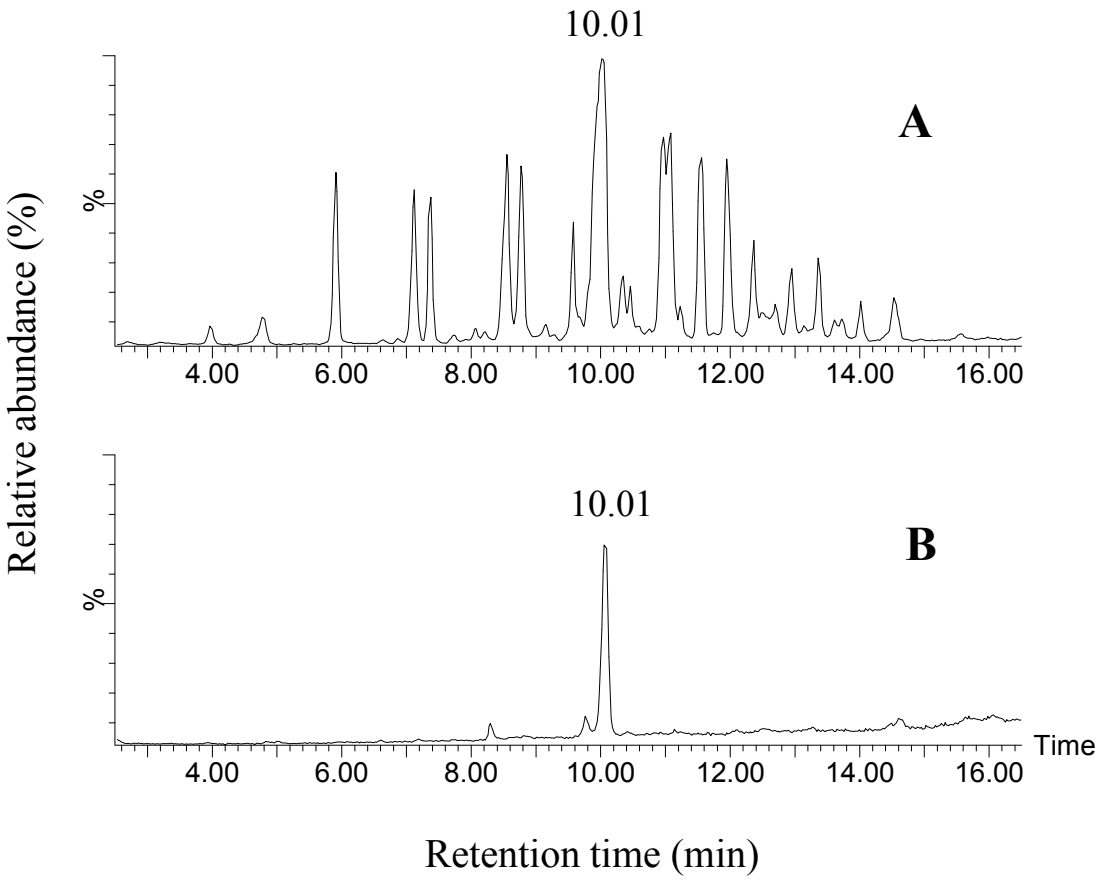
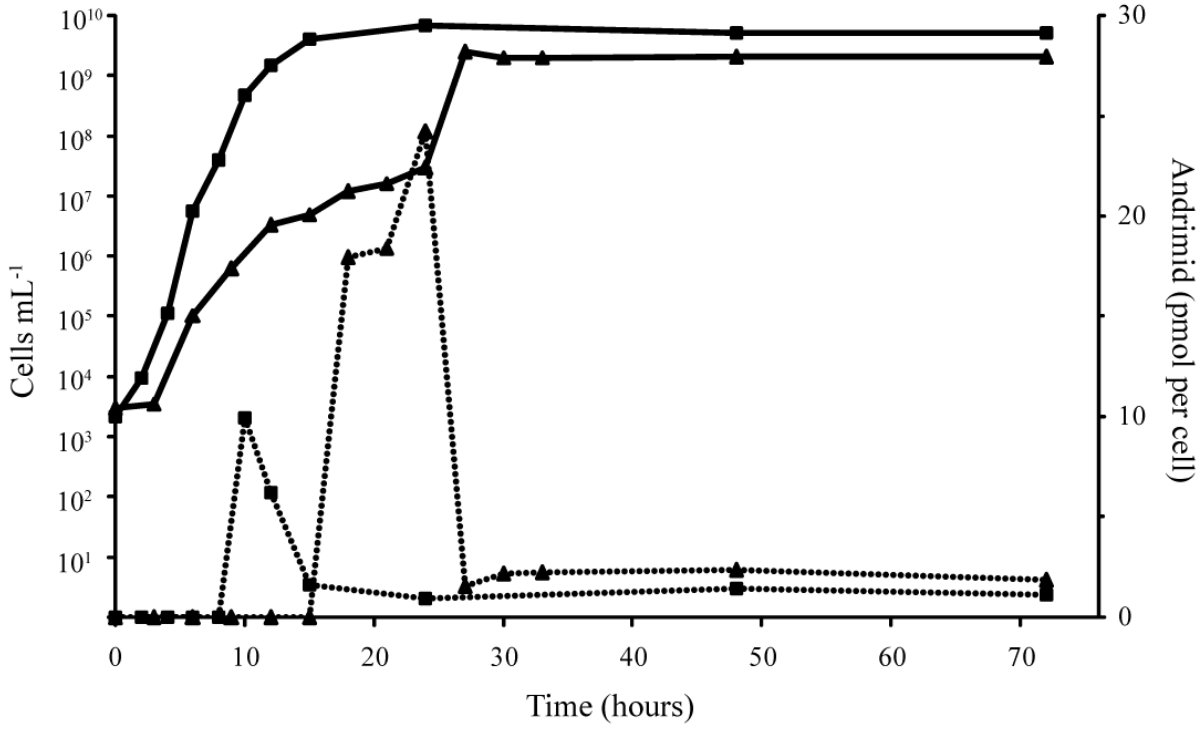
Figure legends

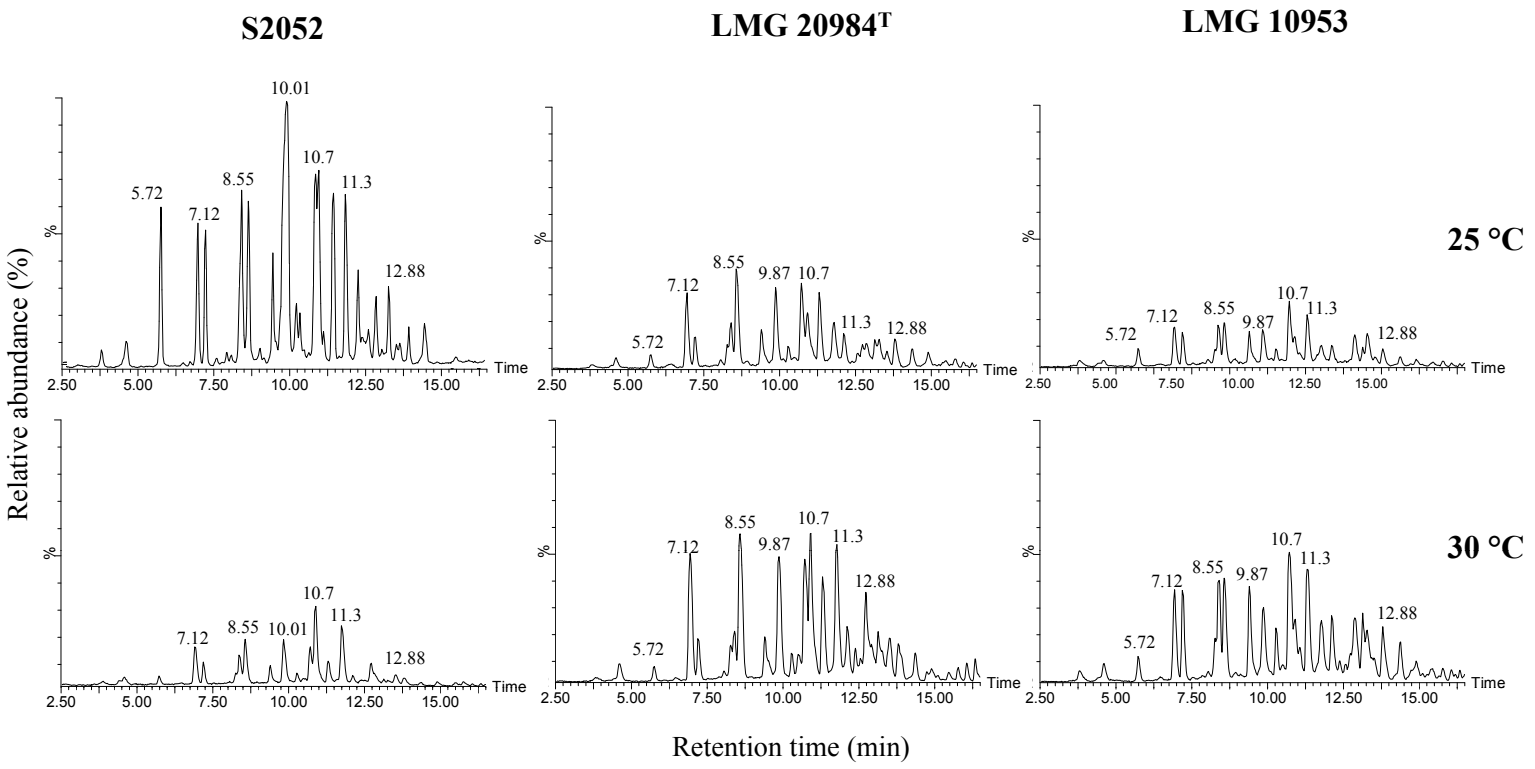
Fig. 1 LC-MS metabolite profiles (total ion chromatogram ESI^+) of *V. coralliilyticus* S2052 in laboratory (A) and chitin (B) medium. The peak with retention time Rt = 10.01 relates to andrimid (Wietz et al., 2010).

Fig. 2 Growth and production of andrimid in *V. coralliilyticus* S2052 over 72 h in laboratory versus chitin medium. Closed lines: cell density (CFU mL^{-1}), dotted lines: yield of andrimid (pmol per cell) in laboratory (■) and chitin (▲) medium.

Fig. 3 LC-MS metabolite profiles (total ion chromatogram ESI^+) of *V. coralliilyticus* S2052, $LMG20984^T$ and LMG10953 grown at 25 and 30 °C.


10.01
A
%
4.00
6.00
8.00
10.00
12.00
14.00
16.00
10.01
B
%
Time
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Relative abundance (%)
Retention time (min)


Cells mL-1
Andrimid (pmol per cell)
Time (hours)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
30
20
10
0
0
10
20
30
40
50
60
70


S2052
LMG 20984T
LMG 10953
25 °C
30 °C
Relative abundance (%)
Retention time (min)
%
Time
5.72
7.12
8.55
9.87
10.01
10.7
11.3
12.88
2.50
5.00
7.50
10.00
12.50
15.00

Paper 6

"Inhibition of Virulence Gene Expression in *Staphylococcus aureus* by Novel Depsipeptides from a Marine *Photobacterium*"

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Ready-to-submit draft for Applied and Environmental Microbiology

Inhibition of Virulence Gene Expression in *Staphylococcus aureus* by Novel Depsipeptides from a Marine *Photobacterium*

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++ Supplemental material for this article may be found at http://aem.asm.org

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Abstract

During a global research cruise, more than five hundred marine bacterial strains capable of inhibiting the growth of pathogenic bacteria were collected. The purpose of the present study was to determine if these marine bacteria are also a source of compounds that interfere with bacterial quorum sensing (QS) systems. In the human pathogen *S. aureus*, the Agr QS system controls a diverse set of virulence factors. Using a gene reporter fusion bioassay, we searched for compounds that interfere with Agr as characterized by enhanced expression of *spa*, encoding Protein A, and reduced expression of *hla*, encoding α-hemolysin, and RNAIII, the effector molecule of Agr. Bioassay-guided fractionation of crude extracts from a marine *Photobacterium* led to the isolation of two novel cyclodepsipeptides, designated solonamide A and B, that repress Agr in *S. aureus* strain 8325-4. Northern blot analysis confirmed the Agr interfering activity of pure solonamides in both *S. aureus* strain 8325-4 and the highly virulent, community-acquired strain USA300 (CA-MRSA). To our knowledge, this is the first report of inhibitors of the Agr system produced by a marine bacterium. (178 words)

Key words: *Photobacterium*, *Vibrionaceae*, antivirulence, quorum sensing inhibition, *agr*, solonamide

Introduction

Microorganisms are an attractive source of new natural products with antimicrobial properties (2,8), and the marine environment constitutes a prolific resource for the isolation of less exploited microorganisms (11,19,51). Many marine microenvironments stimulate the production of specific metabolites as a response to environmental factors (12). It is likely that some of these compounds mediate both intra- and interspecies microbial interactions, including antagonism, and they can therefore be seen as potential new scaffolds for development of drug lead candidates (12,28). The increasing problem of antibiotic resistance among human pathogens highlights the need for novel therapeutic strategies (65). The search for new avenues in microbial control has therefore been extended from traditional growth inhibiting compounds to compounds that target, for example, quorum sensing (QS) pathways (9,52,54). Quorum sensing inhibitors (QSI) do not necessarily kill or inhibit the growth of a pathogen but rather modulate microbial phenotypes, for example by attenuating virulence (3,6).

Staphylococcus aureus is one of the main causes of nosocomical infections, and methicillin-resistant *S. aureus* (MRSA) are emerging at an alarming rate (18,21). The virulence of *S. aureus* is ascribed to a number of virulence factors, including extracellular toxins such as α-hemolysin encoded by *hla*, and cell surface adhesion factors such as Protein A encoded by *spa* (7). Their expression is coordinated through several key regulators, of which the Agr (accessory gene regulator) quorum sensing system is central (44). Agr is encoded by the *agr* locus and is composed of a classical two component system with a sensor histidine kinase, AgrC, and a response regulator, AgrA, in addition to AgrB and AgrD which are responsible for the production of the quorum sensing signal (36,43). Agr-dependent quorum sensing is mediated via cyclic thiolactone octapeptides, termed autoinducing peptides (AIP), that control virulence gene expression through

the effector molecule, RNAIII (24,25). Structure-activity studies (SAR) of the AIPs have led to the construction of synthetic inhibitors of the Agr system (30,36); however, there are only few reports of natural antagonists of this system (26,38,50). Nielsen et al (40) recently developed a screening assay based on *S. aureus lacZ* reporter fusion strains, where the effect of compounds or extracts on expression of three key virulence genes (*spa*, *hla*, and *rnaIII*) and hence potential interference with the Agr locus can be assessed. Subsequently, the assay was used to identify two xanthones as novel quorum sensing interfering compounds in *S. aureus* (40).

We recently established a global collection of marine bacteria that display antibacterial activity (17). The purpose of the present study was to screen pure cultures, crude bacterial extracts, and purified secondary metabolites from this collection of marine bacteria for potential inhibitors of the Agr system. One of the bacterial families investigated was the *Vibrionaceae*. These bacteria are ubiquitous in marine and brackish environments and often associated with marine organisms (61). The *Vibrionaceae* consist of eight genera, with *Vibrio* and *Photobacterium* constituting the majority of species. *Vibrio* spp. can be pathogenic to humans (9,16,34) or marine animals (36), but also occur in the commensal microflora of zooplankton (61) or live as bioluminescent symbionts with squid or fish (15,20,53). The *Photobacterium* genus similarly comprises symbiotic (1,4) and fish pathogenic species (32,62) but does not, to our knowledge, contain any human pathogens. Members of the *Vibrionaceae* have been found to produce broad range inhibitory compounds (17,28), however only few of the antibacterial compounds have been isolated to date. Antimicrobial compounds from *Vibrio* species include the pyrrolidinediones andrimid (29,46,63) and moiramide B (39) that inhibit fatty acid synthesis (49). In addition, we recently reported the production of the potent pyrrothine antibiotic holomycin by a marine *Photobacterium* (63).

Herein, we report the isolation and chemical investigation of two novel depsipeptides produced by that same *Photobacterium* strain. The compounds, designated solonamides A and B, inhibit the Agr quorum sensing system of *S. aureus* and therefore interfere with its virulence gene expression. This indicates that the *Vibrionaceae* and other marine bacteria could be a promising source of novel chemistry with potential use in antibacterial therapy.

Materials and Methods

Isolation and identification of strain S2753. Bacterial strain S2753 was isolated from a mussel surface collected in the tropical Pacific (9.1° S 156.8° E) during the Danish Galathea 3 expedition (17). S2753 was assigned to the *Vibrionaceae* by 16S rRNA gene sequence similarity (17) and identified as being closely related to *Photobacterium halotolerans* based on *recA* and *rpoA* gene sequences, with homologies of 87% (*recA*) and 94% (*rpoA*) (63). BLAST analyses showed that other closely related species were *Photobacterium rosenbergii* and *Photobacterium angustum* S14 (13).

Initial screening for anti-virulence compounds. S2753 was cultured in each 30 mL of (i) marine minimal medium (MMM) (48) containing 0.4% glucose and 0.3% casamino acids, (ii) Marine Broth 2216 (MB; Difco 2216), (iii) Sigma Sea Salts (SSS; Sigma S9883; 40 g L^{-1}) containing 0.4% glucose and 0.3% casamino acids, and (iv) MMM containing 0.2% colloidal chitin prepared according to Wietz et al. (submitted) (64). All cultures were incubated aerated (200 rpm) for 72 hours at 25 °C. All cultures were extracted with an equal volume of ethyl acetate (EtOAc). The extract was evaporated under nitrogen until dryness and redissolved in 300 µL 80% v/v ethanol (EtOH) in water. 20 µL of the extract was tested in an agar-diffusion assay where expression from promoters of *hla*, *rnaIII*, and *spa* is monitored (41).

For further screening, the culture broth of S2753 grown in MMM (1 L, 72 h, 25 °C, 100 rpm) was extracted directly with sterile Diaion HP20SS (12 g L^{-1}, 24 h) (Sigma-Aldrich, St. Louis, MO). The resin was filtered off and washed with 80% (v/v) acetonitrile (MeCN)/water (300 mL) and the extract evaporated until dryness on a rotary evaporator. From this extract, 10 mg dry material was subjected to an explorative solid-phase extraction (E-SPE) protocol by Månsson et al

(33). This yielded 15 fractions for re-testing in the agar-diffusion assay as described above. The E-SPE fractions were also tested for antibacterial activity against *Vibrio anguillarum* strain 90-11-287 and *S. aureus* 8325 in a well diffusion agar assay (22).

Extraction and purification of solonamides A and B. Using 10 L glass fermentors, S2753 was cultured in 5 x 4 L SSS containing 0.4% glucose and 0.3% casamino acids in (25 °C, 72 h, 100 rpm). The broth was extracted directly with Diaion HP20SS (12 g L^{-1}) as described above. The extract (3.4 g) was redissolved in EtOAc, absorbed onto 5 g Isolute diol (Biotage, Uppsala, Sweden) and added to a glass column with pure diol (95 g). A total of 12 fractions were collected from the diol column (100 g, 20 x 350 mm) ranging from heptane, dichlormethane (DCM), EtOAc, to pure methanol (MeOH), running under gravity. The fractions containing the QSI compounds (fraction 5, 20:80 (v/v) EtOAc/DCM and fraction 6, 40:60 (v/v) EtOAc/DCM) were separated on Sepra ZT C_{18} (Phenomenex, Torrance, CA)(10 g SNAP) on an Isolera automated flash system (Biotage) using a MeCN/water gradient 25-75% over 20 min (12 mL min^{-1}). Pure compounds were obtained directly: Solonamide A (17 mg) and B (201 mg). Activity of pure compounds was tested in the agar-diffusion assay as described above (20 µL per well), with a final concentration of compounds of 5 mg mL^{-1} in dimethyl sulfoxide (DMSO).

Structural characterization of the solonamides. NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a 5 mm probe using standard pulse sequences. The signals of the residual solvent protons and solvent carbons were used as internal references (δ_H 2.49 and δ_C 39.5 ppm for DMSO).

LC-MS and LC-MS/MS analyses were performed on a maXis quadrupole time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source. The MS was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA) equipped with a diode-array detector. Separation was performed at 40 ºC on a 150 mm × 2.1 mm ID, 2,6 µm Kinetex C_{18} column (Phenomenex) using a linear water/MeCN (both buffered with 20 mM formic acid) gradient starting from 15% MeCN and increased to 100% in 13 minutes at a flow of 0.4 mL min^{-1}. The MS and MS/MS experiments were performed in ESI^+ with a data acquisition range of *m/z* 100-1200 with collision energy of 27 V. The MS was calibrated using sodium formate automatically infused prior to each analytical run, providing a mass accuracy of less than 1 ppm in MS mode and 2 ppm in MS/MS mode.

The absolute configuration of the amino acids were found using acid hydrolysis (6 M HCl, 110 °C, 20 h) (16) and derivatisation with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA, Sigma-Aldrich) following the protocol described by Bonnard et al. (5). Ultra-high liquid chromatography-diode array (UHPLC-UV) analyses of the amino acids were done on a Dionex RSLC Ultimate 3000 (Dionex) equipped with a diode-array detector. Separation was obtained on a Kinetex C_{18} column (150 x 2.10 mm, 2.6 µm, Phenomenex) maintained at 60 ºC using a linear gradient starting from 25% MeCN in water (both buffered with 50 ppm TFA) increasing to 27% MeCN over 6 minutes at a flow rate of 0.8 mL min^{-1}. Retention times of the FDAA amino acid derivatives used as standards were as follow (maximum standard deviation ± 0.002 min): FDAA (1.50 min), L-Ala (1.14 min), D-Ala (1.61 min), L-Phe (3.58 min), D-Phe (5.04 min), L-Leu (3.77 min), D-Leu (5.49 min), comparable to the observed retention times from the solonamide-derived amino acids.

157 To specify the stereochemistry of enantiomeric amino acids, the depsipeptides were
158 reduced by $LiBH_4$. The resulting linear peptides were subjected to the above mentioned acid
159 hydrolysis and Marfey's derivatisation. Details are given in the Supplemental Material.

160 For the absolute configuration of the fatty acid residues, the (*R*)- and (*S*)-Mosher's
161 esters were prepared for both depsipeptides, and the stereocenters were assigned based on their ^{1}H
162 and ^{19}F chemical shift differences ($\Delta\delta^{SR}$) (10,56). Details are given in the Supplemental Material.

163 *Solonamide A*: white amorphous solids; UV (MeCN/H_2O) λ_{max} 200 nm (100%); for
164 NMR data refer to table 1 in Supplemental Material; HRESIMS *m/z* 558.3416 (calcd for
165 $C_{30}H_{46}N_4O_6$, 558.3496).

166 *Solonamide B*: white amorphous solids; [UV (MeCN/H_2O) λ_{max} 200 nm (100%); for
167 NMR data refer to table 2 in Supplemental Material; HRESIMS *m/z* 586.3725 (calcd for
168 $C_{32}H_{50}N_4O_6$, 586.3730).

169

170 **LC-UV/MS analyses of related *Photobacterium* strains.** To investigate the potential production
171 of Agr inhibitors in related *Photobacterium* strains, the metabolite profile of S2753 was compared
172 by liquid chromatography-diode array/mass spectrometry (LC-UV/MS) to *P. halotolerans* (LMG
173 22194^T), *P. rosenbergii* (LMG 22223^T), and *P. angustum* (S14) described by de Nys et al. (13). All
174 strains were grown in 30 mL MMM containing 0.4% glucose and 0.3% casamino acids for 72 h at
175 25 C and 200 rpm. Cultures were extracted with an equal volume of EtOAc and evaporated under
176 nitrogen. Residues were redissolved in MeOH for LC-UV/MS analyses and in 80% EtOH for
177 biotesting. Inhibition of Agr was tested as described above. Extracts were also tested against *V.*
178 *anguillarum* strain 90-11-287 for inhibition of growth. LC-UV/MS analyses were performed on an

Agilent 1100 liquid chromatograph with a diode array detector (Agilent, Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK) using a Z-spray ESI source. Separation was obtained on a Luna II C_{18} column (50 x 2 mm, 3 µm, Phenomenex) fitted with a security guard system using a linear gradient starting from 15% acetonitrile (MeCN) in water (both buffered with 20 mM formic acid) increasing to 100% MeCN over 20 minutes at a flow rate of 0.3 mL min^{-1}.

Northern blot analysis. Northern blot analysis was performed as described previously (23). The strains used were *S. aureus* FPR 3757 (14), CA-MRSA USA300 obtained from ATCC (Boras, Sweden), and 8325-4 (44). Samples for RNA purification were taken from cultures in Tryptone Soya Broth (TSB, Oxoid, Greve, Denmark) shaking at 185 rpm at 37˚C in a water bath (10 mL culture in 100 mL Erlenmeyer flask). Growth was monitored by measuring optical density at OD_{600}. Start inoculum was OD_{600}=0.03. Solonamides were added at OD_{600}=0.4. Samples for RNA purification were taken at OD_{600}=0.7 and 1.7. Probes targeting *rnaIII*, *spa*, and *hla* transcripts were amplified by PCR using the primers: *hla* forward (5'- GGG TTA GCC TGG CCT TCA GCC-3'), *hla* reverse (5'- GGG TGC CAT ATA CCG GGT TC-3'), *spa* forward (5'- GGG GGT GTA GGT ATT GCA TCT G-3'), *spa* reverse (5'- GGG GCT CCT GAA GGA TCG TC-3'), *rnaIII* forward (5'- GGG GAT CAC AGA GAT GTG ATG-3'), and *rnaIII* reverse (5'- GGG CAT AGC ACT GAG TCC AAG G-3') (TAG Copenhagen A/S, Denmark). The resulting PCR fragments were 311bp (*hla*), 719bp (*spa*), and 316bp (*rnaIII*), respectively.

Results

Identification of QS inhibitors from *Photobacterium halotolerans.* In an initial search for antimicrobial compounds that inhibit the growth of *Staphylococcus aureus* and *Vibrio anguillarum*, we isolated strain S2753 related to *Photobacterium halotolerans* (17). Subsequently, the known antibiotic, holomycin, was identified as responsible for the antibacterial activity (63). When investigating ethyl actetate extracts of S2753 in an agar-diffusion assay monitoring expression of the *S. aureus* virulence genes *hla, rnaIII*, and *spa* (40), we observed an increased expression of *spa* and decreased expression of *hla* and *rnaIII*. The inverse effect of the extracts on *spa* and *hla*/*rnaIII* expression, respectively, indicated the presence of at least one compound that interferes with the *S. aureus* QS system (44). Secondary screening of the extract by explorative solid-phase extraction (E-SPE) (33) detected the potential QSI activity in a fraction that did not inhibit growth of *S. aureus* (data not shown). In the E-SPE separation, we found that diol was a useful column material for the initial separation of the active components. A large-scale culture (20 L) of S2753 was extracted by Diaion HP20SS. Due to poor solubility, the crude extract was absorbed directly onto the diol and fractionated. Final purification was accomplished by C_{18} reversed-phase separation, resulting in the isolation of two compounds active in the *S. aureus* agar-diffusion assay (**Figure 1**). The activity of these pure (NMR purity) compounds was comparable to the initial activity of the extract, confirming that these compounds are responsible for the changes in gene expression. The compounds did not inhibit growth of *S. aureus* or *V. anguillarum* (data not shown). The active compounds were produced by S2753 under all tested culture conditions, including laboratory media (MB, MMM, and SSS) as well as under conditions mimicking the natural environment, using chitin as sole nutrient source (data shown in Supplemental Material).

Structure elucidation of the solonamides. The solonamides were isolated as white powder with respective molecular formulas $C_{30}H_{46}N_4O_6$ (A) and $C_{32}H_{50}N_4O_6$ (B) as determined by HRMS (1 ppm mass accuracy). Analysis of NMR data characterized the structure of the solonamides as cyclodepsipeptides consisting of four amino acids and a 3-hydroxy fatty acid (**Figure 2**) (for details refer to Supplemental Material). The amino acid composition was elucidated as alanine, phenylalanine, and two leucines for both peptides based on DQF-COSY and HSQC NMR experiments. The spin systems of the amino acids were confirmed through strong and unambiguous H2BC correlations (45) and the carbonyl signals assigned by HMBC correlations. Through careful inspection of the DQF-COSY and H2BC data, solonamide A was found to contain a 3-hydroxyhexanoic acid, while B contains a 3-hydroxyoctanoic acid. Long-range HMBC and NOESY correlation data allowed the sequence of amino acids to be established as fatty acid-Phe-Leu-Ala-Leu. This was corroborated by MS-MS experiments giving the exact molecular formula of the fragments (2 ppm mass accuracy). The signal from one oxygen-bearing carbon with a high carbon shift indicated an ester linkage. Lastly, the ring closure linkage was secured by HMBC correlations from H-3 in the fatty acid moiety to the carbonyl in Leu and a weak NOESY correlation from H-3 to the Leu amide and Hα proton. In total, this accounted for the ten degrees of unsaturation resulting from the ring, five carbonyls, and the Phe aromatic ring.

Absolute configurations of the individual amino acid units were established by acid hydrolysis, Marfey's, and UHPLC-UV analysis. Both peptides were found to contain L-Phe, D-Ala, and an enantiomeric pair of L-Leu and D-Leu. Acid hydrolysis of the reduced, linear peptides and subsequent Marfey's derivatisation specified the stereochemistry of the two Leu, exchanging the L-Leu peak (RT 3.77 min) with a new peak (RT 3.73 min), attributable to the corresponding alcohol. Thus, L- and D-stereochemistry was assigned as fatty acid-L-Phe-D-Leu-D-Ala-L-Leu in both solonamide A and B.

The absolute configuration of the fatty acid was found by NMR spectroscopic analysis of the ^{1}H and ^{19}F chemical shift differences ($\Delta\delta^{SR}$) in the (*R*)- and (*S*)- Mosher's esters analysis of solonamide A and B. The absolute stereochemistry of C-3 in the 3-hydroxy fatty acids was established as (*R*) in both depsipeptides (Figure 2).

Solonamides interfere with Agr. Northern Blot analysis was used to verify that the purified solonamides did in fact cause changes in gene expression as observed in the agar-diffusion assay. To this end we isolated mRNA from *S. aureus* 8325-4 and the community acquired strain, USA300, at different stages of growth following solonamide exposure. Solonamide B reduced dramatically the expression of both *hla* and *RNAIII* while increasing expression of *spa*, suggesting that the compound interfere with Agr regulation (**Figure 3**). Intriguingly, the decreased expression of the *RNAIII* effector molecule was even more pronounced in the highly virulent USA300 strain, FPR3757, where high Agr activity is suspected to be a main contributor to the aggressiveness of the strain (14,27). Solonamide A was able to increase *spa* expression, but caused only a marginal reduction of *hla* and RNAIII expression in both 8325-4 and USA300. The discrepancy between results from the Northern blot and the agar-diffusion assay may be rooted in the much higher concentrations of solonamides that are used in the agar-diffusion assay as compared to the Northern blot analysis. Also, the Northern blot analysis requires inspection after ½ and 2 hours, whereas the agar-diffusion assay has a longer time-line for inspection at 15 and 35 hours, which could be of significance for the results.

Production of solonamides by related *Photobacterium* strains. To test whether the solonamides are also produced by related *Photobacterium* strains, we compared S2753 with *P. halotolerans*

LMG 22194^{T}, *P. rosenbergii* LMG 22223^{T}, and *P. angustum* S14 (13). None of these strains produced solonamides (as confirmed by LC-UV/MS), and none affected virulence gene expression in the gene-reporter agar-diffusion assay. None of the three strains inhibited growth of *V. anguillarum*, and holomycin, the antibiotic of S2753 (63), was not detected.

Discussion

The rapid, worldwide increase in antibiotic resistant *Staphylococcus aureus* strains (18) has led to an intense search for compounds with potential use in alternative therapeutic strategies (9). Virulence of *S. aureus* involves a complex set of proteins, with the Agr quorum sensing system controlling expression of several of the virulence genes. The investigation of crude extracts and fractions from a marine *Photobacterium* led to the identification of two novel depsipeptides, solonamides A and B, as potent inhibitors of this system. The natural inducers of Agr are 16-membered thiolactone macrocycles carrying a peptide tail necessary for full Agr activation through binding to the membrane bound sensor histidine kinase (31,36). Structure-activity studies demonstrated that the macrocyclic ring is required for antagonistic activity (31), and synthetic studies have led to the construction of global inhibitors based on truncated AIPs (31,37). The similar structures and sizes of the solonamides and the truncated AIPs (**Figure 4**) suggest that they may be competitive inhibitors of the Agr system. Unlike the AIPs, the solonamides are cyclized through a 3-hydroxy fatty acid forming a lactone rather than a thiolactone. However, synthetic lactone and lactam variations of natural AIPs have been found to have antagonistic activity (31,35,36), which our study corroborates. While cross-inhibition of Agr by AIPs is more tolerant of sequence and structural diversity than is activation (31), Mayville et al (35) found that the presence of the hydrophobic leucine and phenylalanine residues is important for the inhibition of the Agr response. Both solonamides contain a leucine and phenylalanine; however, the reduced activity of solonamide A suggests that the overall hydrophobicity of the depsipeptides affected by the varying length of the fatty acid moiety is an important factor influencing activity.

Interestingly, we found solonamide B interefered with Agr not only in *S. aureus* 8325-4 but also in strain USA300, which is a predominant community-acquired MRSA (CA-MRSA)

300 strain in the US (60). This finding suggests that quorum sensing inhibition could be an option for
301 treatment of *S. aureus* USA300 infections. The solonamides are the first report of antagonists
302 produced by a natural source mimicking the structure of natural AIPs. There are few other examples
303 of natural antagonists of the Agr system. Kiran et al (2008) identified hamamelitannin from
304 *Hamamelis virginiana* (witch hazel) as an inhibitor of RNAIII and δ-hemolysin expression in *S.*
305 *aureus* 8325-4, USA300, and clinical *S. epidermidis* isolate MH (26). Also, ambuic acid from an
306 unidentified fungal strain was found to attenuate Agr (38).

307 To the best of our knowledge, only two species of *Photobacterium* have been
308 investigated for their chemistry so far (34,47,57). Oku et al (47) isolated the unnarmicins A and C
309 (**Figure 5**) from a marine *Photobacterium* strain MBIC06485 related to *P. leiognathi*. Like the
310 solonamides, unnarmicin A and C consist of four amino acids (L-Phe, L-Leu, D-Phe, L-Leu) and a
311 3-hydroxyoctanoic and 3-hydroxyhexanoic fatty acid, respectively. The unnarmicins were found to
312 have selective antibacterial activity against two strains of *Pseudovibrio* (47) and inhibit the fungal
313 ABC transporter (47,58). Due to the high structural similarity, it can be hypothesized that the
314 unnarmicins are also capable of attenuating Agr. The finding of the unnarmicin in another marine
315 *Photobacterium* sp. suggests that production of such peptides could be a common feature in this
316 group of bacteria. Yet, we did not detect solonamide A or B in any of the related strains that we
317 investigated.

318 The yield of solonamides was up to 5 mg L^{-1}, which is high, compared to other γ-
319 proteobacteria. For example, the yield of unnarmicins from a *Photobacterium* sp. was ten times
320 lower (47). The solonamides were produced on a chitin based medium, mimicking natural
321 conditions, which indicates that these compounds are produced in the natural environment. Also,
322 the solonamides have D-alanine and D-leucine incorporated; amino acids that are not present in the

laboratory medium. Thus, the *Photobacterium* seems to produce these specific stereoforms rather than incorporating available amino acids. Altogether, this suggests that the solonamides are more than mere metabolic bi-products or storage compounds produced under laboratory conditions.

Given the relatively low abundance of staphylococci in the marine environment, it seems unlikely that the *P. halotolerans* S2753 produce solonamides as part of a deliberate strategy to interfere with this specific type of bacteria. However, the solonamides might be targeted at other gram-positive bacteria in the marine environment, such as bacilli and actinobacteria, though little is known about quorum sensing pathways in marine Gram-positive bacteria. Acylated homoserine lactones, the most widely researched type of QS molecules in Gram-negatives, can serve as both agonists and antagonists in different systems (50), and thus the solonamides may also serve as quorum sensing signals for the *Photobacterium* itself. However, we did not detect solonamides or compounds with similar QSI activity in any of the related strains.

QS inhibitors have been found from other marine bacteria. Teasdale et al (2009) identified two phenylethylamides from a *Halobacillus salinus* capable of inhibiting QS-controlled behaviors in gram-negative bacteria (59). In addition, extracts from coral-associated *Bacillus horikoshij* inhibits biofilm formation in *Streptococcus pyogenes* through suspected QS inhibition (42). Blockage of accessory gene functions with such natural inhibitors might promote colonization of surfaces or accession of nutrients (24,36,55). The full extent of the combination of QSI and antibiosis for bacterial competition in the marine environment is still unknown.

Considering their ubiquitous presence in the marine environment, the *Vibrionaceae* are chemically under-explored. However, this study as well as other recent work from our lab (63,64) demonstrate that species within the *Vibrionaceae* are capable of producing secondary metabolites with antibacterial activities. Here we have demonstrated that members of *Vibrionaceae*

346 could be a promising source of new bioactive compounds with nontoxic effects against pathogenic
347 bacteria by the report of three novel cyclic depsipeptides with selective inhibition of the Agr system
348 in *S. aureus*.

349

350 **Acknowledgments.**

351 We thank Anita Iversen for ultra high-resolution MS data. Funding from the Programme Committee
352 for Food, Health and Welfare under the Danish Strategic Research Council is acknowledged. The
353 present work was carried out as part of the Galathea 3 expedition under the auspices of the Danish
354 Expedition foundation. This is Galathea 3 contribution pXX (number to be added if accepted).

355

List of figures:

Figure 1: Effect of solonamides (A and B) on *hla*, *rnaIII* and *spa* expression. Solonamides (5 mg mL^{-1}) were added to wells in TSA plates containing the 8325-4 derived *lacZ* reporter strains PC322 (*hla::lacZ*), SH101F7 (*rnaIII*::*lacZ*), or PC203 (*spa::lacZ*). Incubation time was 15 h for plate I and II, and 35 h for plate III (plate numbering indicated with white letters).

Figure 2: Structures of solonamides A and B produced by *Photobacterium halotolerans* strain S2753.

Figure 3. Effect of solonomides A and B on virulence gene expression in *S. aureus* strain 8325-4 (44) and USA300 (14) examined by Northern blot analysis. Solonamides were added to exponentially growing cultures at OD_{600}=0.4, and RNA was purified at OD_{600}=0.7 and 1.7. The RNA was reacted with probes recognizing *hla, rnaIII*, and *spa*, respectively. DMSO was used as negative control.

Figure 4. (I) Structure of natural group I AIP (R = Thr-Ser-Tyr-H), (II) Synthetic Agr inhibitor based on a truncated AIP-I analogue (37).

Figure 5. Structure of unnarmicin A (R = Et) and C (R = H) (47).

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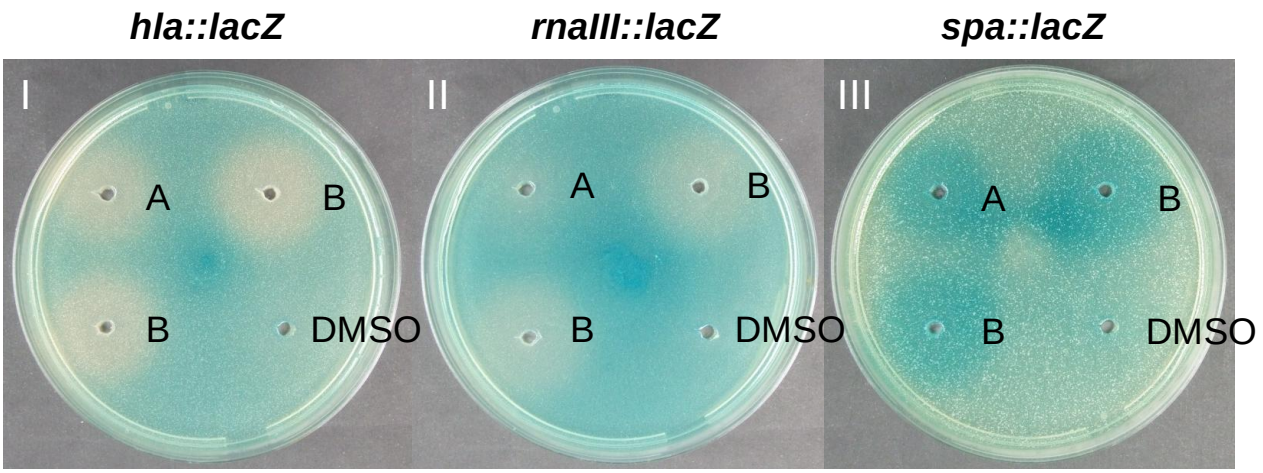

375

376 **Fig 1**

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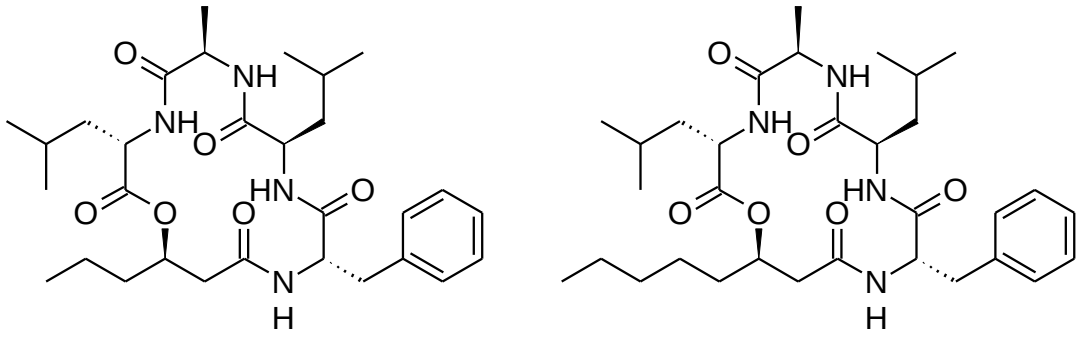

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380 **Fig 2**

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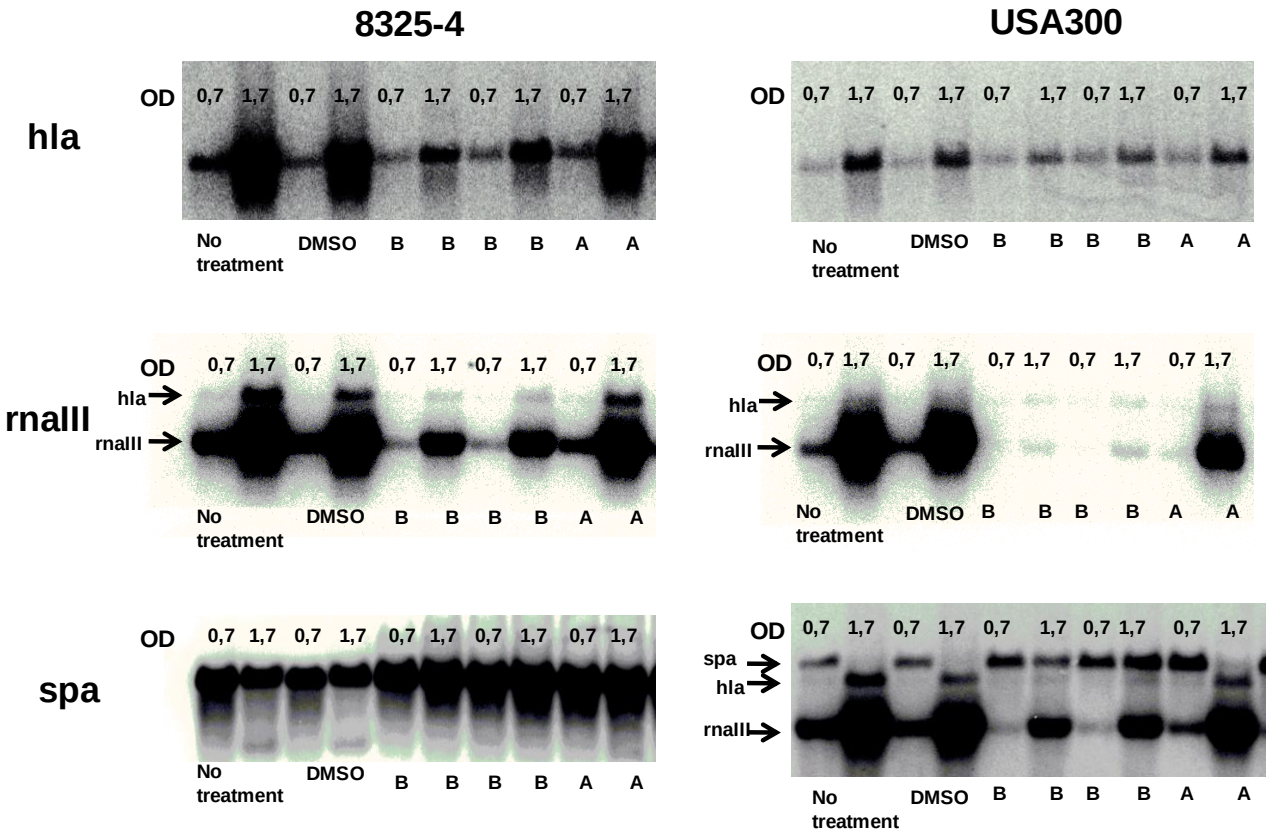

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383 **Fig 3**

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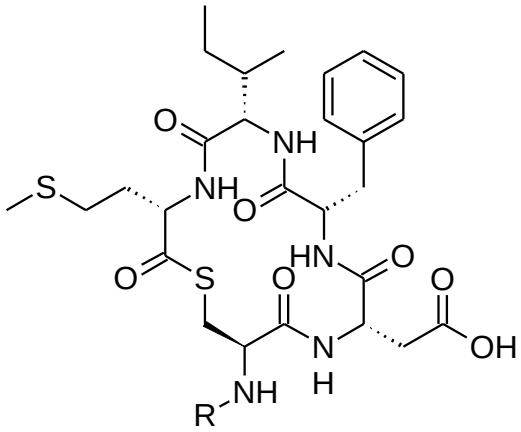

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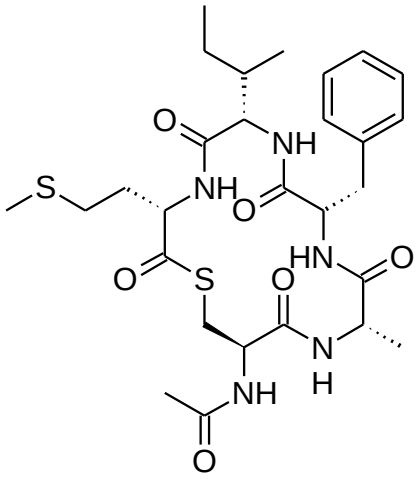

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387 **Fig 4**

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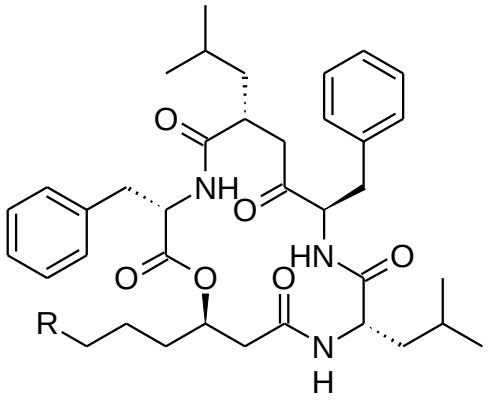

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391

392 **Fig 5**

393

394 Reference List

395

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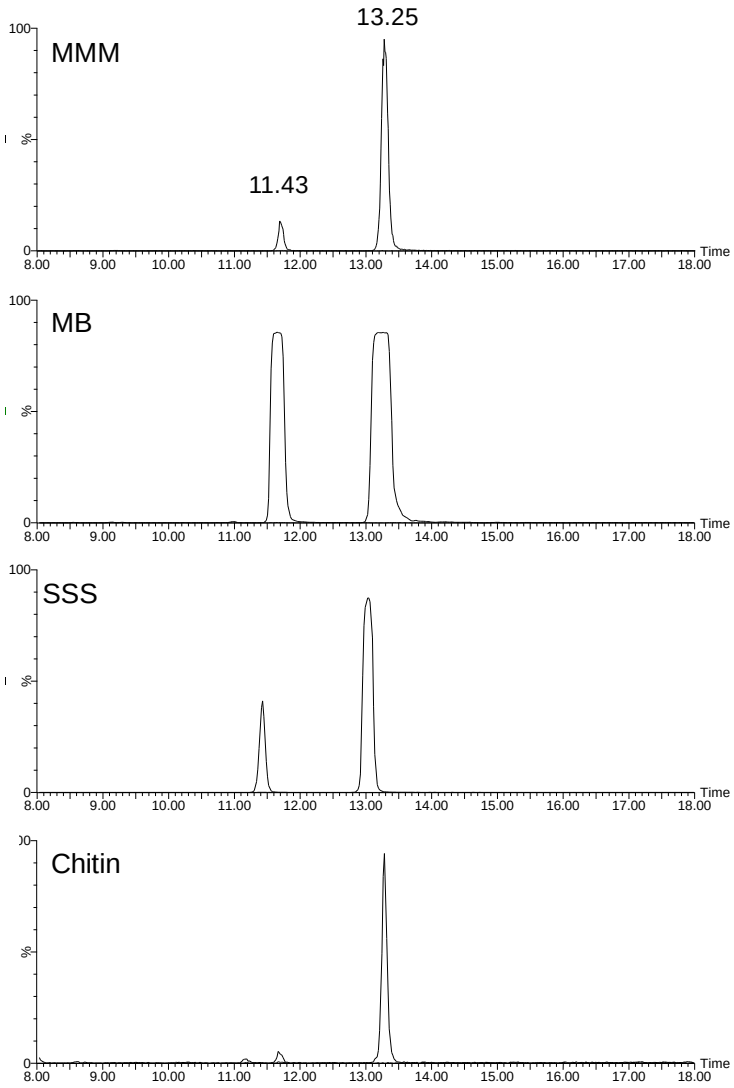
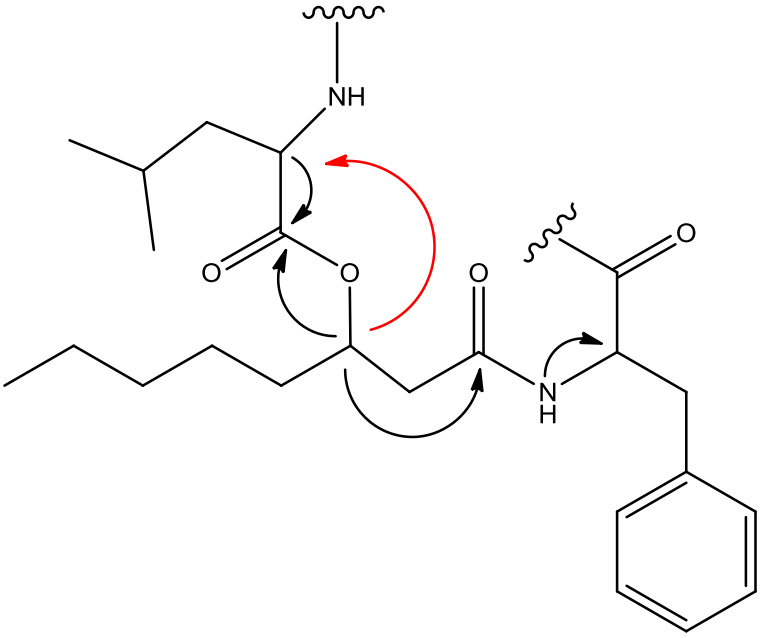
Supplemental Materials

A1: LC-MS selective ion-trace of solonamide A (*m/z* 558, RT = 11.43 min) and solonamide B (*m/z* 586, RT = 13.25 min) production on different growth media, including marine minimal media with glucose (MMM), marine broth (MB), sigma sea salts (SSS), and marine minimal media with chitin (Chitin). Slight shift in retention times due to inter-batch variations on the LC-MS system.



NMR

Assignments of the depsipeptides were performed using a conventional assignment approach. HMBC correlations between 3-H/1-CO, 3-H/36-CO and NOEs between 3-H/31-Hα-Leu confirms the lactone closure of the structure. The figure (**A3**) below shows key correlation for the lactone ring closure.



A4. Numbering scheme used for the assignment of solonamid A (left) and B (right)

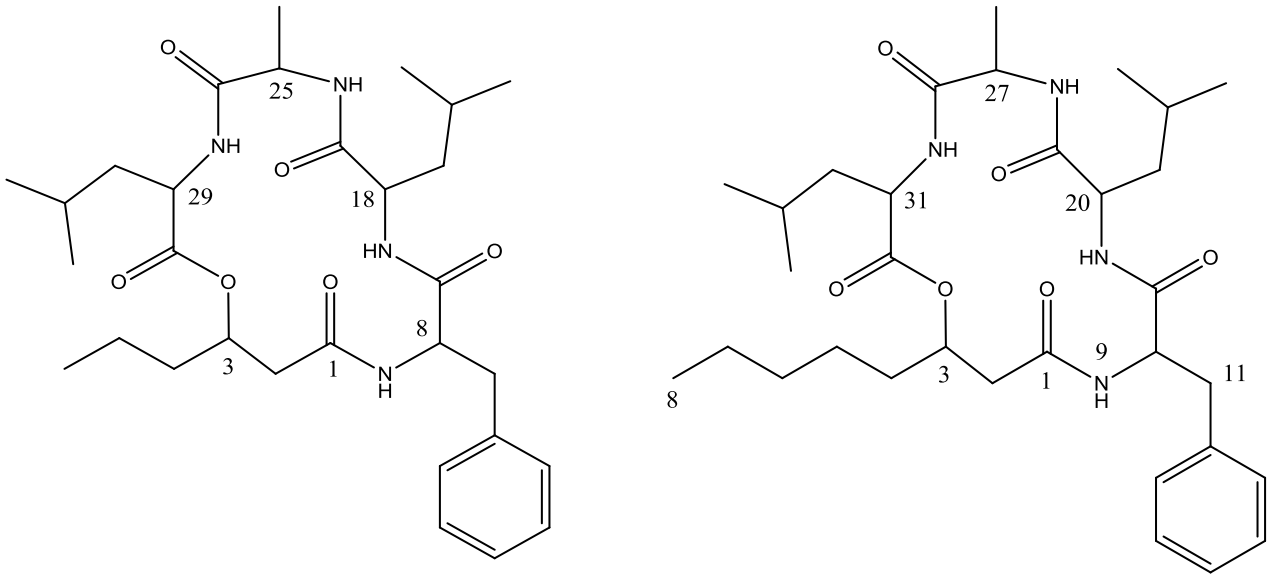


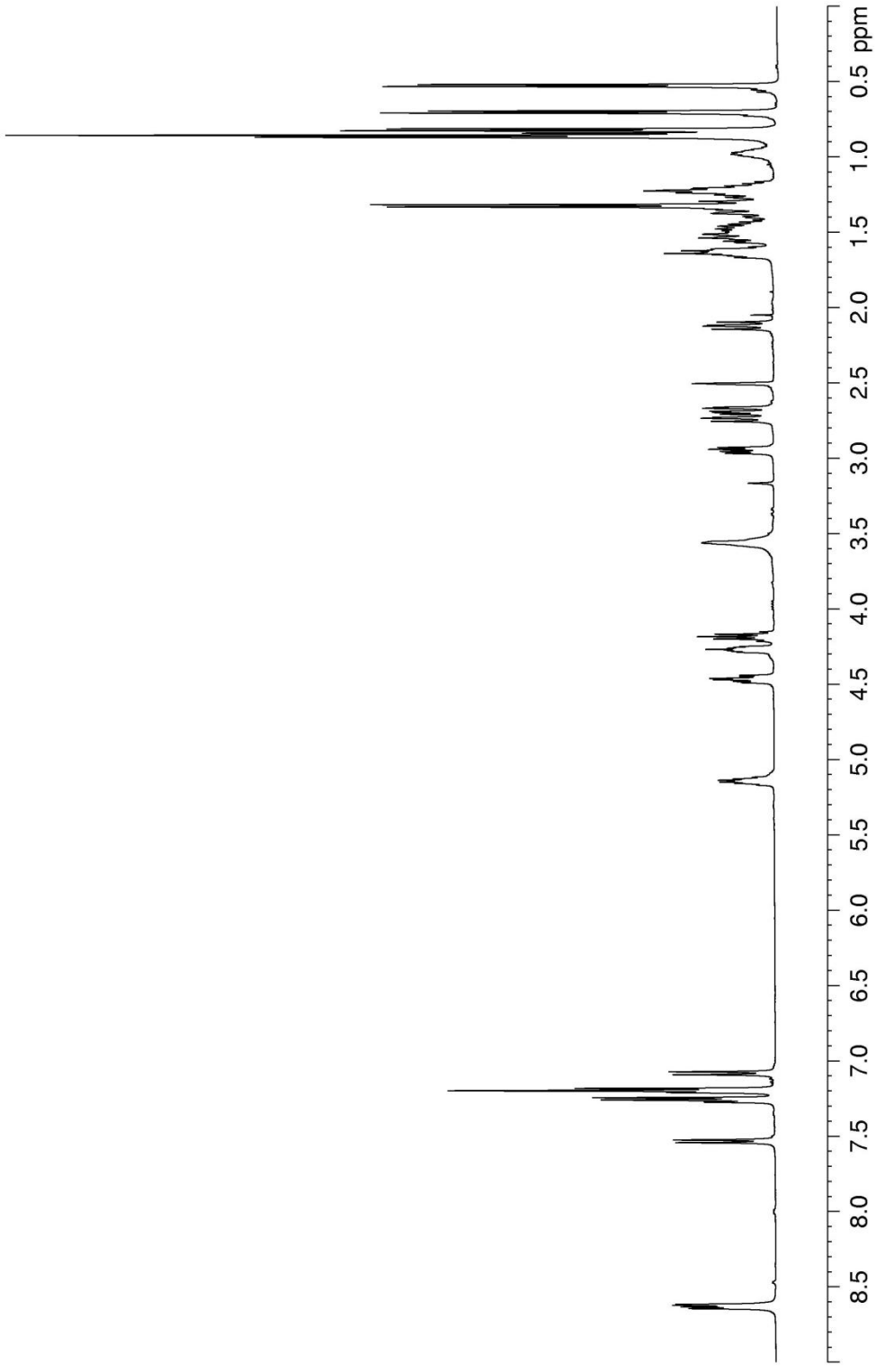
Table 1. Solonamide A

Atom assignment	^{1}H-chemical shift/ppm #H, multiplicity, J/Hz	^{13}C-chemical shift/ppm	HMBC connectivities
Hha			
1	-	170.6	-
2	2.67, 1H, dd, 13.5, 3.8 2.11, 1H, dd, 13.5, 10.1	40.7	1, 3, 4
3	5.14, 1H, m	72.1	1, 5
4	1.62, 1H, m 1.45, 1H, m	36.2	2, 3, 6
5	~1.22, 1H, m	17.7	3, 4
6	0.85, 3H, t, 7	13.8	4, 5
Phe			
7 - NH	8.61, 1H, d, 3.2	-	1
8 - CH_{α}	4.26, 1H, ddd, 10, 6.1, 3.2	55.9	9, 10, 14
9 - $CH_{\beta,1}$	2.94, 1H, dd, 13.3; 6.1 2.72, 1H, dd, 13.3; 10	36.4	8, 10, 11/15, 14
10	-	135.8	-
11,15	7.18, 2H, m	129.1	9, 13
12,14	7.25, 2H, m	128.4	10
13	7.18, 1H, m	126.5	11
16 - CO	-	174.4	-
Leu^{1}			
17 - NH	8.63, 1H, d, 5.6	-	16, 18, 19
18 - CH_{α}	3.56, 1H, m	53.1	
19 - $CH_{\beta,1}$	1.36, 1H, m 1.29, 1H, m	38.9	18, 21, 22
20 - CH_{γ}	0.97, 1H, m	23.5	21
21 - $CH_{\delta,1}$	0.69, 3H, d, 6.6	23.2	19, 20
22 - $CH_{\delta,1}$	0.52, 3H, d, 6.5	20.7	19, 20
23 - CO	-	171.8	-
Ala			
24 - NH	7.53, 1H, d, 8.9	-	23,25
25 - CH_{α}	4.18, 1H, m	48.2	26, 27
26 - $CH_{\beta,1}$	1.32, 3H, d, 7.4	16.6	27
27 - CO	-	171.3	-
Leu^{2}			
28 - NH	7.08, 1H, d, 10	-	27, 29
29 - CH_{α}	4.46, 1H, dt, 10; 4.4	49.0	30, 31, 34
30 - $CH_{\beta,1}$	1.63, 1H, m 1,53, 1H, m	39.3	29, 31, 33, 27/34
31 - CH_{γ}	1.50, 1H, m	23.9	29, 30, 33
32 - $CH_{\delta,1}$	0.86, 3H, d, 6.5	23.2	30, 31, 33
33 - $CH_{\delta,1}$	0.81, 3H, d, 6.5	21.3	30
34 - CO	-	171.2	-

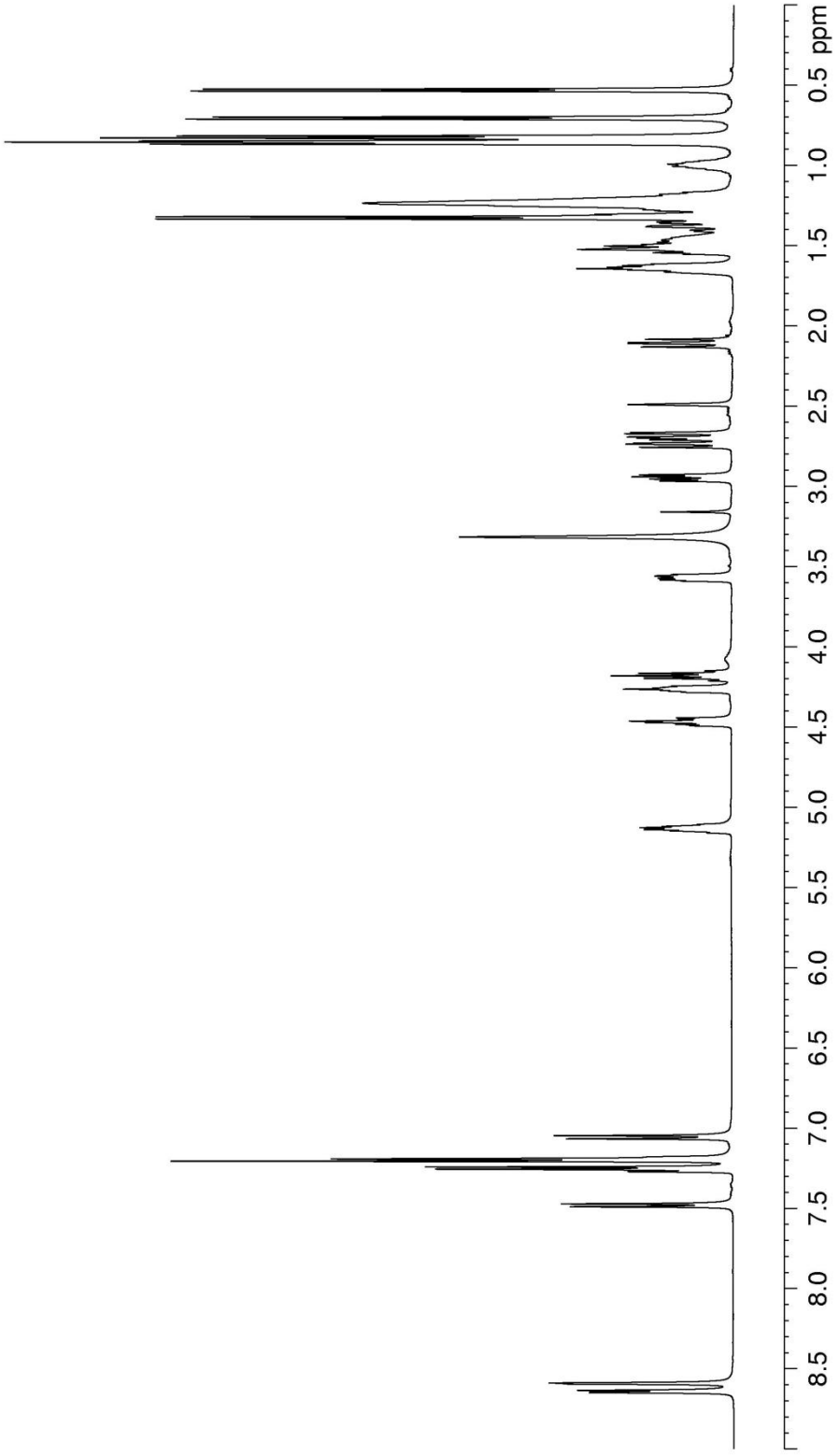
Table 2. Solonamide B

Atom assignment	^{1}H-chemical shift/ppm #H, multiplicity, J/Hz	^{13}C-chemical shift/ppm	HMBC connectivities
Hoa			
1	-	170.4	-
2	2.69, 1H, dd, 13.5; 3.7 2.11, 1H, dd, 13.5; 10.3	40.5	1,3,4
3	5.13, 1H, m	72.0	1, 4, 5, 36
4	1.65, 1H, m 1.45, 1H, m	33.8	5, 6
5	~1.20, 2H, m	23.8	6,7
6	~1.22, 2H, m	30.8	
7	1.25, 2H, m	21.8	
8	0.84, 3H, m	13.6	6
Phe			
9 - NH	8.59, 1H, d, 3.0	-	1, 10, 11, 18
10 - CH_{α}	4.26, 1H, ddd, 10;6.1; 3.3	55.6	11,18
11 - CH_{β}	2.95, 1H, dd, 13.3; 6.1 2.73, 1H, dd, 13.3; 10	36.1	10,12, 13/17,18
12	-	136.0	-
13,17	7.20, 2H, m	128.7	11, 15
14,16	7.26, 2H, m	128.0	12, 14/16
15	7.20, 1H, m	126.2	13/17
18 - CO	-	174.2	-
Leu^{1}			
19 - NH	8.64, 1H, d, 5.6	-	18, 20, 21
20 - CH_{α}	3.57, 1H, m, 9; 5.6; 3.5	52.7	21, 22, 25
21 - CH_{β}	1.31-1.37, 2H, m	38.6	20, 22, 24
22 - CH_{γ}	0.99, 1H, m	23.3	
23 - $CH_{\delta,1}$	0.70, 3H, d, 6.6	22.9	21, 22, 24
24 - $CH_{\delta,2}$	0.53, 3H, d, 6.4	20.4	21, 23
25 - CO	-	171.6	-
Ala			
26 - NH	7.48, 1H, d, 8.9	-	25, 27, 28
27 - CH_{α}	4.18, 1H, m	47.8	28, 29
28 - CH_{β}	1.33, 3H, d, 7.3	16.4	27, 29
29 - CO	-	170.9	-
Leu^{2}			
30 - NH	7.05, 1H, d, 10	-	29, 31
31 - CH_{α}	4.47, 1H, dt, 10; 4.3	48.6	32, 33, 29/36
32 - $CH_{\beta,1}$	1.64, 1H, m 1.52, 1H, m	39.0	31, 33, 35, 29/36
33 - CH_{γ}	1.50, 1H, m	23.8	32, 34,35
34 - $CH_{\delta,1}$	0.86, 3H, d, 6.6	23.0	32, 33, 35
35 - $CH_{\delta,1}$	0.82, 3H, d, 6.4	21.1	32, 33
36 - CO	-	170.8	-

A5. 1D ^{1}H spectrum of Solonamide A

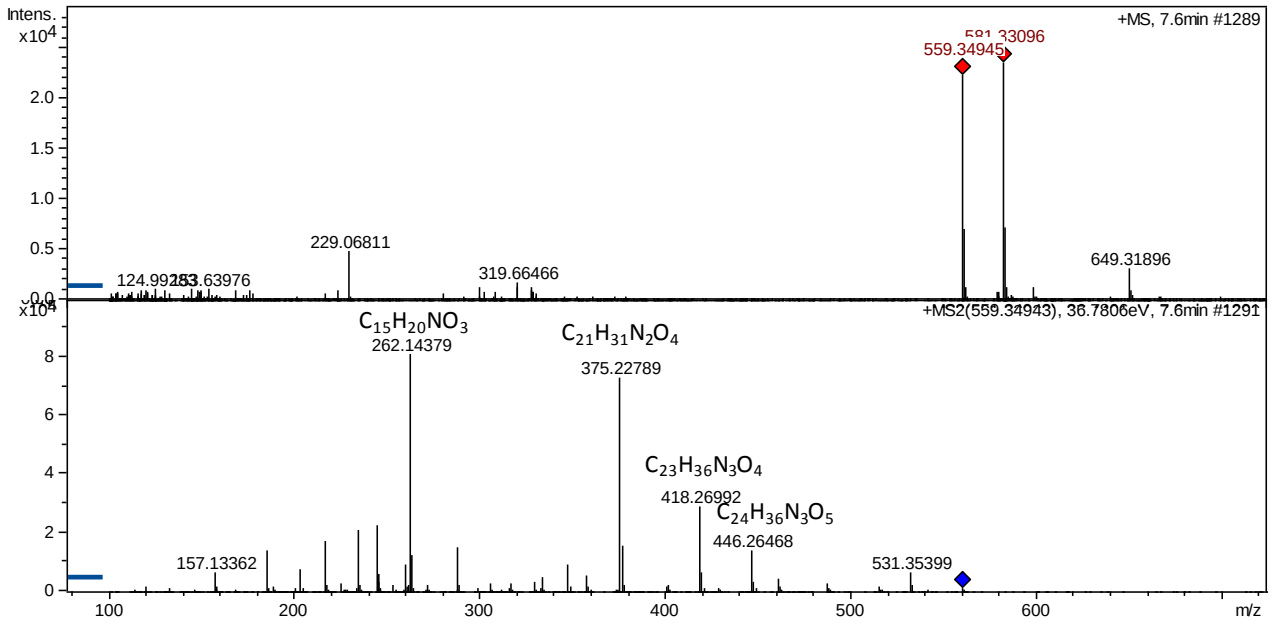


A6. 1D ^{1}H spectrum of Solonamide B

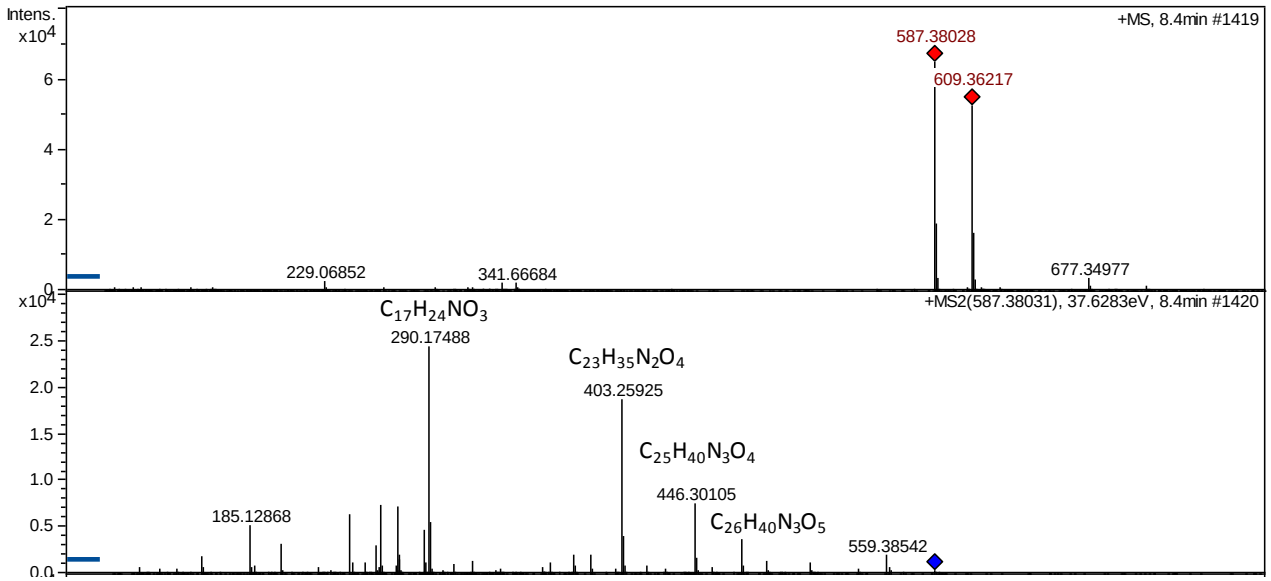


MS/MS data

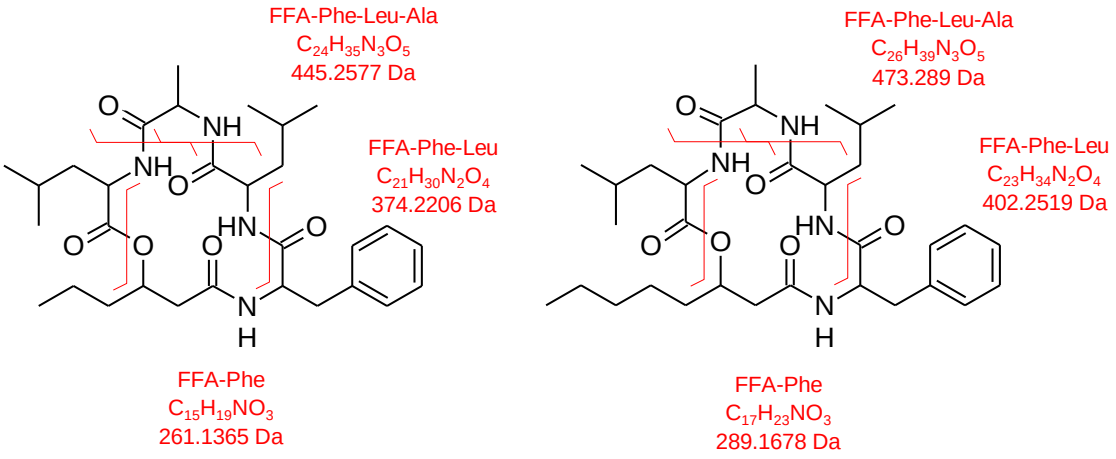
A7: MS and MS/MS spectrum of solonamide A ($C_{30}H_{46}N_4O_6$, calcd. 558.3496, Δ *m/z* 0.8 ppm) with characteristic fragments and their molecular formulas (Δ *m/z* 0.2-0.6 ppm):



A8: MS and MS/MS spectrum of solonamides B ($C_{32}H_{50}N_4O_6$, calcd. 586.3730, Δ *m/z* 0.0 ppm) with characteristic fragments and their molecular formulas (Δ *m/z* 0.2-0.6 ppm):



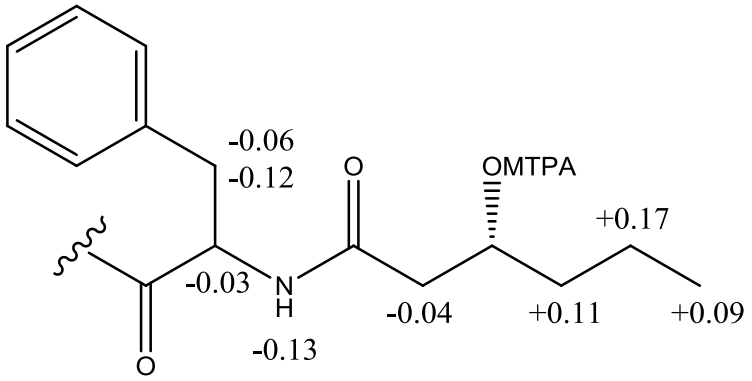
A9: Characteristic fragments for solonamide A (left) and B (right):



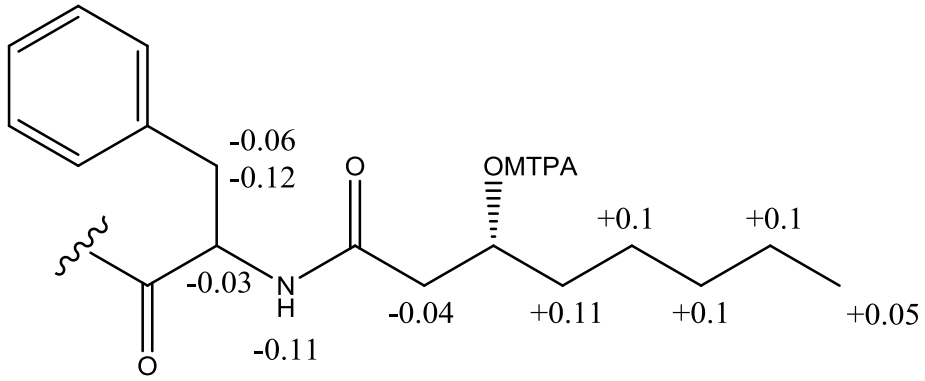
Establishment of absolute configuration

The absolute configuration of C3 at 3-hydroxyhexanoic acid (Hha) and 3-hydroxyoctanoic acid (Hoa) was determined using the Mosher's method. Here with the Mosher's method two enantiomers of an acid chloride (*α*-methoxy-*α*-trifluoromethylphenylacetic acid chloride, MTPA-Cl) are reacted with the secondary alcohol at the stereogenic center in order to reveal the configuration of this center. The reaction yields two diastereoisomers with known configuration around one of the stereogenic centers enabling the determination of the other stereogenic center.

The sign of the Δδ values can be seen in the figures below, all pointing toward the same configuration at the C3 stereocenter in Hha and Hoa. The values for Δδ are positive for the alkane end of Hha/Hoa and negative towards the carbonyl end, which points towards an *R*-configration of the C3 stereocenter. The difference seen in the flourine NMR data supports this conclusion when the alkane chain of the molecule is regarded as least bulky compared to the remaining part of the molecule with a Δδ(SR) ~ -0.68 ppm .



A10. Distribution of the Δδ values calculated for the 3-hydroxyhexanoic acid (Hha) of solonamide A



A11. Distribution of the Δδ values calculated for the 3-hydroxyoctanoic acid (Hoa) of solonamide B

Experimental procedure used for Mosher's:

Methanolysis of solonamide A (and B)

5 mg (8.52 μmol) of the depsipeptide (solonamide A) is dissolved in 1.5 mL of 0.5 M NaOMe in MeOH and stirred at room temperature for 1 h. The reaction mixture is neutralised by careful addition of 1M HCl (aq) and the pH is monitored with universal indicator paper. The reaction mixture is concentrated to dryness on a rotary evaporator, and partitioned in EtOAc and H_2O. Methanolysis of solonamide B was performed using the same procedure. The organic phase is

concentrated to dryness on a rotary evaporator and the methyl ester product verified by LC-MS ($[M+H]^+$ A: 591.3803 Da, B: 619.4053 Da) and purified by RP-HPLC on a Waters HPLC with a 600 controller and a 996 photodiode array detector using a water/MeCN gradient elution from 40 to 60 % MeCN over 20 min (5 mL/min) with a Luna C_{18} column (5 µm, 250x10 mm, Phenomenex). Pure methyl esters of solonamide A (1.1 mg) and B (2.7 mg).

Preparation of the *R*-MTPA ester of solonamide A (and B)

To a stirred solution of the purified methanolysis product (0.55 mg, 0.889 µmol) in dry pyridine (150 µL) is added 5 µL (catalytic amount, 0.1 eq) of a solution of dimethylaminopyridine (DMAP) in dry pyridine (0.4 mg, 50 µL). After 10 min 9.1 µL *S*-(+)-MTPA-Cl (48.6 µmol) is added and the reaction is left overnight at room temperature. The reaction mixture is dried on a speedvac, redissolved in MeOH and purified on a Waters HPLC with a 600 controller and a 996 photodiode array detector using a water/MeCN gradient elution from 45 to 100 % MeCN over 20 min (5 mL/min) with a Luna C_{18} (5 µm, 250x10 mm, Phenomenex) column to give pure *R*-MTPA ester (A: 0.5 mg, B: ~1.2 mg).

Preparation of the *S*-MTPA ester of solonamide A (and B)

Using the same procedure as for the *R*-MTPA ester, the *S*-MTPA ester is produced by reaction with *R*-(–)-MTPA-Cl. Pure *R*-MTPA esters (A: 0.4 mg, B: ~1.1 mg).

Establishing stereochemistry of enantiomeric Leu by reduction and Marfey's

2 mg of solonamide B is reduced in 2 M $LiBH_4$ in THF at 0°C for 30 min, then at rt overnight and lastly heated to 50˚C for 30 min. EtOAc is added and the reaction is quenched with sat. NH_4Cl (aq) before the product is extracted with EtOAc, CH_2Cl_2 and Et_2O concentrated and purified by RP-HPLC on a Waters HPLC with a 600 controller and a 996 photodiode array detector using a water/MeCN gradient elution from 25 to 80% MeCN over 20 min (5 mL/min) with a Luna C_{18} column (5 µm, 250x10 mm, Phenomenex). Yields 0.7 mg of reduced open-chain solonamide B. The same procedure is used for solonamide A, only with a gradient of 35-70 %, yielding 0.8 mg.

300 µg of each peptide is hydrolysed with 300 µL 6 M HCl at 110°C for 20 h. To the hydrolysis products is added 75 µL water, 20 µL 1 M $NaHCO_3$ solution and 100 µL 1% FDAA in acetone, followed by reaction at 40°C in 1 h. The vial is removed from the heat, neutralised with 10 µL 2 M HCl and the solution is diluted with 395 µL MeOH to a total volume of 0.5 mL. The FDAA derivatives are analysed by UPLC on a Dionex Ultimate 3000 with a diode array detector and a Kinetex C_{18} column (2.6 µm, 150x2.10 mm, Phenomenex). The analyses are run with a gradient elution of water/MeCN from 25 to 37 % MeCN over 6 min (60°C, 0.8 mL/min) and the FDAA derivatives of the hydrolysates are compared to retention times of the standard amino acid derivatives: D-Ala (1.61 min), L-Ala (1.14 min), D-Leu (5.49 min), L-Leu (3.77 min), D-Phe (5.04 min), L-Phe (3.585 min). FDAA elutes at 1.50 min.

Appendix A

Table 1 Reported bacterial metabolites in AntiBase 2010 according to their genus, class, and phylum.

Phylum	Class	Genus	No. of compounds
Actinobacteria	'Marine actinobacteria'	*Streptomyces*	326
		Actinomyces	121
		Micromonospora	20
		Microbacterium	18
		Salinospora	11
		Janibacter	9
		Actinomadura	8
		Nocardiopsis	7
		Micrococcus	6
		Brevibacterium	2
		Chainia	1
Proteobacteria	γ- proteobacteria	*Vibrio*	82
		Alteromonas	59
		Pseudomonas	4
		Pseudoalteromonas	26
		Halomonas	14
		Marinobacter	10
		Aeromonas	9
		Photobacterium	7
		Shewanella	2
		Enterobacter	2
		Deleya	1
	α-proteobacteria	*Rhodobacter*	27
		Agrobacterium	15
		Ruegeria	13
		Roseobacter	3
		Oceanibulbus	3
		Pelagiobacter	3
		Erythrobacter	1
		Blastobacter	1
	δ-proteobacteria	*Myxobacteria*	18
	β-proteobacteria	*Chromobacterium*	15
		Alcaligenes	2
Firmicutes	Bacilli	*Bacillus*	52
		Halobacillus	5
		Enterococcus	1
Cyanobacteria	'Marine cyanobacteria'		112
Bacteroidetes		*Cytophaga*	20
		Flavobacterium	7
		Chryseobacter	2
		Cyclobacterium	1
		Flexibacter	1
Chloroflexi	Chloroflexi	*Chloroflexus*	2
Unidentified bacteria			68

b

Appendix A